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**MAMMALIAN CELL
HYBRIDIZATION: II**



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MAMMALIAN CELL HYBRIDIZATION: II

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PREFACE

Mammalian cell hybridization involves the fusion of the plasma membrane from two individual cells. It is striking that cell fusion can even be accomplished between cells from different species of animals. As a consequence of this fusion the individual nuclei from the two cells now share the same cytoplasm and in turn there will be a mixing of the two genomes following cell division of the hybrid. This phenomena of cell fusion became experimentally exploited when it was discovered that Sendai virus possess a closely associated substance termed "fusion factor". Other viruses have subsequently been shown capable of fusing cells, although Sendai virus has remained the agent of choice for the procedure. Recently, there has been data presented which suggests that fusion of cells can be accomplished in the absence of virus.

Although the chemical nature of the "fusion factor" as well as the mechanism of cell fusion has not been precisely defined, this technique has provided the means for studying numerous properties of somatic cells. This collection of articles is a survey of certain areas where cell hybridization has been utilized. The process of fusion itself is reviewed and the usefulness of hybridization for the rescue or activation of viruses from cells which are infected but which may not be expressing the agent. Also included are studies aimed at deciphering how a virus becomes intimately associated with the host cell genes and the consequence of this association with respect to whether the cell will become diseased or not.

Perhaps one of the more significant events that take place following the fusion of mouse and man cells is the preferential loss of human chromosomes from the hybrid. This phenomena has opened up a route for mapping genes in man. These latter studies which are included in this collection, have been greatly aided due to the difference in the karyotype of mouse and human chromosomes. In addition the gene products such as enzymes and cell membrane components are different between the two species and are readily distinguishable. Many of the articles touch on the possibility of fusing specialized cells, which have a precise function with an undifferentiated cell in order to ascertain the selective expression of the genetic information contained within that cell. This fact may provide a means to decipher gene control in higher organisms. And lastly this collection reflects the body of literature that is available on the usefulness of all hybrids

Many of the articles touch on the possibility of fusing specialized cells, which have a precise function with an undifferentiated cell in order to ascertain the selective expression of the genetic information contained within that cell. This fact may provide a means to decipher gene control in higher organisms. And lastly this collection reflects the body of literature that is available on the usefulness of all hybrids to analyze malignancy of a cell.

Ronald T. Acton, Ph.D.
January, 1973

Viruses and Cell Fusion

Cell Fusion without Viruses

By J. A. LUCY, Q. F. AHKONG, F. C. CRAMP, D. FISHER and J. I. HOWELL.

Aqueous dispersions of lysolecithin (lysophosphatidylcholine) can induce cell fusion and the formation of both homokaryons and heterokaryons (Poole, Howell & Lucy, 1970). Electron microscopy has now shown that fusion of avian erythrocytes by lysolecithin involves the establishment of small cytoplasmic bridges between cells. The polykaryocytes subsequently produced disintegrate rapidly owing to generalized membrane damage, but damage can be minimized by using lysolecithin in micro-droplets of glycerol trioleate. Only those regions of membrane in contact with the lipid droplets are then directly affected, and the droplets may be regarded as morphological equivalents of Sendai-virus particles. These experiments may be relevant to membrane fusion in the exocytosis of milk-fat droplets containing lysolecithin (Keenan, Morré, Olson, Yunghans & Patton, 1970).

The following procedure has been devised to determine the ability of defined chemicals to cause cell fusion. Hen erythrocytes in Eagle's medium or buffered saline at pH 5.6 containing Dextran 60C (mol.wt. 77500) (80 mg/ml) are treated with the test substances at 37°C for up to 2 h, and bovine serum albumin is then added. Lysis accompanying cell fusion is thus minimized, except with aqueous

lysolecithin, and the polykaryocytes remain intact for 24 h. This allows the quantitative measurement of cell fusion by optical and electrical methods. Various degrees of cell fusion are seen with retinol, oleic, linoleic, linolenic, lauric acid glycerol mono-oleate, glycerol monolaurate and selachyl alcohol, and when Ca^{2+} ions are followed by F^{-} ions. Fusion apparently does not occur in this procedure with glycerol monopalmitate, glycerol monostearate, palmitic, stearic and elaidic acids.

Fusion of avian erythrocytes in Eagle's medium by glycerol mono-oleate is inhibited by serum albumin and by EDTA; Ca^{2+} ions facilitate fusion. Glycerol mono-oleate will also fuse hen erythrocytes with mouse LS fibroblasts. In preliminary experiments on cell hybrids formed from mouse 3T3TK⁻ fibroblasts and wg3IMP⁻ hamster fibroblasts, clones of viable cells occur in HAT medium (Littlefield, 1963) 4-7 times more frequently with glycerol mono-oleate (50 µg/ml) than in untreated cultures. As glycerol mono-oleate damages cells less than lysolecithin, it may be the more useful compound for cell fusion without viruses in somatic genetics.

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Growth of Newcastle Disease and Herpes Virus and Interferon Production in a Monkey-Mouse Hybrid Line

By J. COPPEY

INTRODUCTION

By fusing human and mouse cells, Wang obtained a hybrid line which was permissive for poliovirus as were the human parental cells (Wang *et al.* 1970). Similarly, a mouse-hamster line was shown to be permissive for polyoma virus as were the mouse parental cells (Basilico, Matsuya & Green, 1970). The ability of these hybrid lines to support the multiplication of polio and polyoma viruses was further shown to be due to the part of the chromosomal complement from the permissive parental lines. We present here a similar situation for a monkey-mouse hybrid line infected with either Newcastle Disease virus (NDV) or herpes virus, which were the only viruses so far tested which multiply at high levels in both the monkey parental and the hybrid cells as compared with the mouse parental cells. Because NDV is a potent inducer of interferon, it seemed to us interesting to study the levels and characteristics of the interferon produced by these hybrid cells as well as of those produced by the parental lines.

METHODS

Cells. CV-1, an established line of green monkey kidney cells (Jensen *et al.* 1964) and MKS-B, a SV40 transformed strain of mouse kidney cells (Dubbs *et al.* 1967) were used respectively between passage 50 and 80, and passage 150 and 200. By fusing these lines, Kit *et al.* (1970) developed a hybrid cell line, MK-CV^{III}. Clone 4 of this hybrid (Cassingena *et al.* 1971) was used between passages 29 and 60. It was always subcultured with a medium containing HAGT (see Littlefield, 1965). This clone at passage 27 contained a few monkey

chromosomes and almost the total complement of mouse chromosomes (Cassington *et al.* 1971). For the duration of experiments, HAGT was omitted from the medium.

Viruses. Stocks of Sindbis (Aegypt A 39) were prepared on primary chick embryo cells (CE), those of vaccinia (Joklik) and vesicular stomatitis virus (VSV, Indiana Strain) on L cells, NDV (L Kansas 48) in the allantoic fluid of 11-day-old eggs and that of herpes (Schealey) on CV-1. Viruses were titrated either on CE (Sindbis, VSV, NDV) or on CV-1 cells (herpes, vaccinia).

Anti-NDV and anti-herpes sera were obtained from hyperimmunized rabbits.

Virus growth was performed in 1-day-old tube cultures (10^5 cells for seeding) or in 2 to 5 days old cultures (according to the cell type) in Petri dishes (10^6 cells for seeding). The virus was adsorbed at 37° for 60 min.; the inoculum volume was 0.3 ml./dish or 0.1 ml./tube. The non-adsorbed virus was measured after mixing excess inoculum with five successive washings of the monolayers. Virus yield was measured after three cycles of freezing and thawing in the infected cultures and after removal of cell debris by centrifugation. Titres are given in p.f.u.

Infectious centres. Cells were infected (m.o.i. = 3) rinsed 3 times with P.B.S., kept for 30 min. at 37° in the presence of antiserum (diluted 1 to 10), then trypsinised and plated on CE.

Interferon. The supernatants of infected cells (m.o.i. = 3) were collected 48 hr after infection, kept at pH 2.3 at $+4^\circ$ for 6 days, ultracentrifuged ($100,000 g$ for 2 hr) then tested for their interferon content in MA 104 cells (monkey interferon) and in L cells (mouse interferon) as previously described (Coppey & Markovits, 1969). The action of either interferon was strictly species specific: for example we obtained 256 and 4096 units respectively of monkey and mouse interferon with one preparation which titred less than 2 when tested in the opposite system.

Mol. wts of interferons were determined by gel filtration on G-100 columns, 30×1.8 cm. Samples of 1 ml. of 20 times concentrated interferons were applied and mol. wts were estimated according to Andrews (Andrews, 1964).

U.v. irradiation was done on confluent monolayers of cells after removal of the medium, using a Philips germicidal tube delivering $25 \text{ ergs mm.}^{-2} \text{ sec.}^{-1}$.

RESULTS

Virus growth

Virus production was measured after a single cycle in cells infected at a m.o.i. of 3 (Table 1). Whereas the yields of VSV, Sindbis and vaccinia viruses are comparable in the three cell lines, the yields of NDV and of herpes are about 50 times less in MKS-B than in both CV-1 and hybrid cells. To ensure that these differences observed in the titres of NDV and of herpes only reflects differences in virus multiplication, cells were infected at the low m.o.i. of 0.01. Virus yields were measured 3 days later, i.e. after several successive cycles of virus multiplication (Table 1). NDV and herpes multiplication occurred in MKS-B cells, but to a level 1000 times lower for NDV and about 20 times lower for herpes as compared with the two other lines. The ability of hybrid cells to support multiplication of both viruses was found to be a stable property between passages 29 and 63 of subcultivation.

These observed differences in the yield of both viruses using MKS-B, CV-1 and MK-CV^{III} clone 4 cells were not attributable to differences in the virus adsorption (Table 2). The percentage of infected cells which produce NDV was comparable in the three lines, and

that of infected cells producing herpes was 8 to 10 times less in MKS-B than in CV-1 and hybrid cells (Table 2).

From these data we calculated that the average yield of NDV was about 0.1 p.f.u./cell in MKS-B, 100 p.f.u./cell in CV-1 and 70 p.f.u./cell in MK-CV^{III} clone 4, whereas the yield of herpes was about 2 p.f.u./cell in MKS-B, and 160 p.f.u./cell in CV-1 or in MK-CV^{III} clone 4 cells.

Table 1. *Growth of different viruses in the different cell lines*

Viruses...	m.o.i. of 3; 24 hr yield (p.f.u./10 ⁵ cells)*					m.o.i. of 0.01; 72 hr yield (p.f.u./10 ⁵ cells)*	
	VSV	Sindbis	Vaccinia	NDV	Herpes	NDV	Herpes
Cell type							
CV-1	6.3 × 10 ⁷	3 × 10 ⁸	1.2 ± 0.5 × 10 ⁶	2.8 ± 0.5 × 10 ⁶	4.2 ± 0.7 × 10 ⁶	4 ± 1.1 × 10 ⁶	2.7 × 10 ⁶
MKS-B	3 × 10 ⁷	8.1 × 10 ⁷	3.6 ± 1.2 × 10 ⁶	4.5 ± 3 × 10 ⁴	7 ± 2 × 10 ⁴	2.3 ± 1.6 × 10 ³	1.1 × 10 ⁶
MK-CV ^{III} clone 4	8.7 × 10 ⁷	7 × 10 ⁸	3.5 ± 1.1 × 10 ⁶	2 ± 0.4 × 10 ⁶	3.1 ± 0.9 × 10 ⁶	1.6 ± 0.5 × 10 ⁶	1.3 × 10 ⁶
CV-1 LL E46†	—	—	—	3.1 × 10 ⁶	3.8 × 10 ⁶	—	—
LM (TK ⁺)§	—	—	—	2 × 10 ⁶	4.3 × 10 ⁶	—	—
LM (TK ⁻)‡	—	—	—	1.8 × 10 ⁶	4.1 × 10 ⁶	—	—

* Standard deviations, if given, were calculated from four to five separate experiments.

† Line of CV-1 transformed by an irradiated hybrid virus Adeno 7-SV40.

§ Mouse L cells.

‡ Mouse L cells deficient in thymidine kinase activity

Table 2. *Virus adsorption and percentage of cells yielding virus after infection with NDV or herpes*

Cell type	Virus adsorption (% of inoculum)		Infectious centres (% of plated cells)	
	NDV	Herpes	NDV	Herpes
CV-1	72	75	33 ± 8	27.5 ± 3
MKS-B	70	66	23 ± 2	3.1 ± 1.3
MK-CV ^{III} clone 4	75	67	35 ± 5	17 ± 2.1

U.v. sensitivity of the capacity for NDV and herpes production

To define the role of the monkey part of the hybrid line in supporting the multiplication of NDV and herpes, we irradiated the cells with u.v. just before infection, and measured the subsequent virus yield. Our purpose was to try to dissociate by u.v. the DNA directed cell functions concerned with the capacity to support virus growth, because the DNA is the main target of u.v. and we hoped that u.v. would modify virus production in MKS-B and CV-1 cells differently. This was the case for NDV, as clearly shown in Table 3: u.v. irradiation of the cells up to 1000 ergs mm⁻² increases the virus yield in MKS-B but decreases it in both CV-1 and in MK-CV^{III} clone 4 at the same rate. Therefore, the capacity of hybrid cells exhibits exactly the same u.v. sensitivity as that of CV-1, which is quite different from that of MKS-B. On the other hand, the u.v. sensitivity of the ability of the three cell lines to support herpes growth are all different, MKS-B being the most resistant and MK-CV^{III} clone 4 the most sensitive (Table 3).

Interferon production

It has already been shown that different hybrid lines produce two types of interferon after infection by NDV. These interferons carry the same species specificity as those of the parental lines (Guggenheim, Friedman & Rabson 1968; Carver, Seto & Migeon, 1968). The same double interferon producing response to NDV was found for MK-CV^{III} clone 4 (Cassingena *et al.* 1971).

Table 3. *U.v. sensitivity of the cell capacity to support virus growth*

Virus Dose of u.v. ergs mm ⁻² ...	NDV					Herpes			
	0	125	250	500	1000	0	250	500	1000
Cell type									
CV-1	100	88	67	45	11	100	16	1.7	0.1
MKS-B	100	240	195	180	175	100	30	8	1.1
MK-CV ^{III} clone 4	100	90	65	43	13	100	0.6	0.023	0.0060

Medium was poured off from cultures (2×10^6 cells per Petri), cells were irradiated with a Philips germicide tube (dose rate of $25 \text{ ergs mm}^{-2} \text{ sec}^{-1}$), then infected at m.o.i. of 3. Viruses were harvested 24 hr later. Virus yields of irradiated cultures are expressed as a percentage of yields from the unirradiated cultures (mean of two separate experiments; MK-CV^{III} clone 4 were tested at passages 30 and 36).

Table 4. *Interferon production by the three cell lines*

Interferon inducer...	Sindbis	NDV	poly rI+rC, 5 µg/ml.	RF of mengo virus, 5 µg./ml.
Cell type				
CV-1	8 to 32	128 to 512 (M.W. = 50,000)*	< 2	< 2
MKS-B	128 to 512	2,048 to 8,192 (M.W. = 39,000)*	48 to 64	128
MK-CV ^{III} clone 4: mouse interferon	64 to 512	1,024 to 4,096 (M.W. = 39,000)*	32 to 64	128
MK-CV ^{III} clone 4: monkey interferon	2 to 8	8 to 32 (M.W. = 50,000)*	< 2	< 2

(*) = Molecular weight of samples of NDV induced interferon (for hybrid cells at passage 32). The samples were 20 times concentrated before G-100 filtrations.

We also found that MK-CV^{III} clone 4 produces both high levels of mouse and low levels of monkey interferon after infection by either NDV or Sindbis virus (Table 4): the yield of monkey interferon was roughly 1 % of that of mouse interferon. Using replicative form (RF) of mengo virus RNA (a gift of Dr E. Falcoff) (Falcoff & Falcoff, 1969) or poly rI+rC as inducers, we found that hybrid cells produce mouse interferon, but not monkey interferon, in detectable amounts (Table 4). This last result probably means that the ratio of monkey interferon to mouse interferon is an intrinsic property of these hybrid cells and not of the type of inducer used. Moreover for all these inducers, hybrid cells always produce mouse interferon to the same extent as do MKS-B, whereas they produce 5 to 15 times less monkey interferon than CV-1 cells after infection by NDV or Sindbis. These results are in good agreement with the fact that MK-CV^{III} clone 4 contains only a partial complement of monkey chromosomes and almost the full complement of mouse chromosomes (Cassingena *et al.* 1971).

The mol. wts of monkey and mouse interferons produced by these hybrid cells at passage 32 as measured on Sephadex G-100 are the same as those from corresponding parental lines (Table 4), namely 50,000 and a slight excluded peak for monkey interferon, and 39,000 for mouse interferon. Furthermore, after re-chromatography on G-100 of each peak from a hybrid interferon preparation it was not possible to show any intermediate peak, which would correspond to a hypothetical 'hybrid interferon' as suggested by others (Carver *et al.* 1968).

DISCUSSION

For two unrelated viruses, NDV and herpes, we have shown that a monkey-mouse hybrid line is as permissive as its parental monkey line, CV-1, whereas its parental mouse line, MKS-B is weakly permissive (small yield of viruses per cell and, moreover, for herpes a small percentage of infected cells yielding virus). This weak permissiveness, although unexplained, is probably not connected with two characters of MKS-B, namely lack of thymidine kinase activity (TK -) and SV40 transformed state (Dubbs *et al.* 1967): we have found for NDV as well as for herpes that (a) LM (TK +) mouse cells are as permissive as LM (TK -), and (b) that the same holds true for CV-1 compared with CV-1 LL 'E 46', a CV-1 transformed line (Table 1).

The u.v. sensitivity of the capacity to support the multiplication of NDV differed between CV-1 and MKS-B cells, but was the same in hybrid cells as in monkey CV-1 cells. Because the cell DNA is the main target for u.v. in the low dose range employed in these experiments (Coppey & Markovits, 1969), the last result indicates that the permissiveness of hybrids for NDV is a property conferred by its monkey chromosomal complement, a conclusion similar to those of Wang *et al.* (1970) for a human-mouse hybrid infected with poliovirus and of Basilico *et al.* (1970) for a mouse-hamster hybrid infected with polyoma virus.

Although the mechanism (s) by which u.v. irradiation of cells modify their capacity to support virus multiplication is unknown, this result also suggests that NDV replicates using at least some of the monkey functions of these hybrids.

We found that NDV infected MKS-B or hybrid cells produce similar amounts of interferon. It was previously shown that the interferon inducing capacity of NDV was probably dependent on the production of a virus replicative intermediate in monkey cells (Coppey & Markovits, 1969), as it is for numerous others RNA viruses (see Hilleman, 1970). Such a replicative intermediate was recently shown in NDV infected cells (Bratt & Robinson, 1971). From this view point, the high production of interferon in MKS-B cells infected with NDV could be a consequence of the arrest of the virus productive cycle at a stage after the synthesis of its replicative intermediate.

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Viral-induced Fusion of Human Cells

II. HETEROKARYOCYTES BETWEEN HUMAN CELLS FROM DIFFERENT DONORS, AND BETWEEN HUMAN CELLS AND THOSE FROM OTHER SPECIES: FORMATION AND PROPERTIES¹

ANTONIO VELÁZQUEZ²

In a previous paper (Velázquez et al., '71) a simple method was described which yields a high frequency of polykaryocytes in cultures of human diploid fibroblasts. This method involves the addition of UV-inactivated Sendai virus to a cell monolayer, without removing the medium, and exposing the cells to the virus for about 12 hours. When this technique is used, the frequency of nuclei which are present in multinucleated cells (the polykaryocytic index) is about 50%. One of the purposes of the present study was to determine the degree of heterokaryocytosis induced by this method, when the virus is added to a mixed culture containing different strains of human diploid fibroblasts, or cultures containing a mixture of human diploid fibroblasts and cultured cells derived from other species. Other reports have described the use of the conventional method (which involves exposing a mixed cell suspension to the virus for a short length of time) for the induction of fusion between different strains of human diploid fibroblasts (Siniscalco et al., '69), or human diploid fibro-

blasts and fowl erythrocytes (Guggenheim et al., '68; Harris and Cook, '69). An additional objective of this study was to inquire whether or not hybrid enzymes could be detected in cultures containing interspecific heterokaryocytes.

MATERIALS AND METHODS

Some of the materials and methods used have been described earlier (Velázquez et al., '71). Only those not previously employed are summarized below.

Composition of culture media

The composition of automedium (Krooth, '64) is similar to nucleomedium (Velázquez et al., '71) with two exceptions: auto-

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medium contains fetal bovine serum which has been dialysed for 21 hours against tap water and for three hours against distilled water. Secondly, it does not contain nucleosides or NCTC 135.

Cell lines

MIS is a human fibroblastic line developed from a skin biopsy on a normal Caucasian adult female. 3T3 was derived from a Swiss mouse embryo (Todaro and Green, '63), and was kindly supplied by Dr. George J. Todaro. CHA₂ was developed from the lung of a male Chinese hamster (V-explant) by Ford and Yerganian ('58). A subline (V79-379A) from the original line was kindly supplied by Dr. Norman Salzman. The R00 line was derived from a male rat-kangaroo (Shaw and Krooth, '64). MIG is a clonal line of human mouse hybrid cells, which was kindly supplied by Dr. Barbara Migeon.

Formation of heterokaryocytes

One of the two cell lines to be fused was labelled with thymidine-methyl-H³ (19.2 c per mM; New England Nuclear Corp., Boston, Mass.) at a final concentration of 0.3 μ Ci/ml for 72 hours. The cultures were then washed twice with a nucleomedium containing non-radioactive thymidine at a concentration of 52.4 mg per liter. This concentration of thymidine is about 10⁴-fold higher than the concentration of the tritiated compound used to label the cells. This modified nucleomedium containing unlabelled thymidine was used throughout the rest of the experiment. After being washed with medium, the cultures were trypsinized, suspended in medium and mixed in a 1:1 ratio with cells of the unlabelled strain. The total concentration of cells in the suspending medium was 4×10^5 per milliliter. One milliliter of this suspension was then added to each of six Leighton tubes and cells were allowed to attach to the surface of the tubes. In addition, six pure cultures of each parental line were plated at the same initial cell density. The cells in each culture were then fused by the procedure described previously (Velázquez et al., '71): half of the cultures were exposed to virus (and half to Hanks' balanced salt solution) for 12 hours and were later subcultured at low population density to Petri dishes con-

taining coverslips. After six to eight hours, the coverslips were fixed, washed in 0.3 N trichloroacetic acid at 4°C for one hour, rinsed several times with distilled water, dipped briefly in 70% ethanol and air-dried. Finally, the coverslips were mounted on slides and autoradiographs were made.

Labelling of cultures with tritiated leucine

DL-leucine- 4,5-H³ (5 c per mM; New England Nuclear Corp., Boston, Mass.) was added to the medium to give a final concentration of 4.12 μ Ci per milliliter. This quantity of radioactivity corresponds to a concentration of 0.108 μ g of leucine per milliliter, which is less than 1/500th the concentration of leucine present in nucleomedium or in complete automedium.

Acrylamide gel electrophoresis

The cells were harvested by trypsinization and were suspended in distilled water and disrupted by sonication (Krooth et al., '62). The cell sonicate was extracted twice with toluene and then centrifuged at 23,000 g for 30 minutes. Sucrose was added and the extracts were dehydrated by vacuum evaporation. The final protein concentration was 10 mg per milliliter and the final sucrose concentration was 20 gm %. The solutions were then frozen and stored at -60°C.

Acrylamide gels were prepared by adding 14 mg of Cyanogum-41, 0.2 ml of TMED (N,N,N',N'-tetramethylethylenediamine) and 200 mg of ammonium persulfate) all from EC-Apparatus Corp., Philadelphia, Pennsylvania) to 200 ml of a buffered solution (*vide infra*). The electrophoretic migration of lactic acid dehydrogenase (LDH) and malic acid dehydrogenase (MDH) in acrylamide gels was measured using a Survey Model Vertical Gel Cell (EC-Apparatus Corp.). The buffered solution used for LDH was tris-Na₂ EDTA-boric acid (0.1 M), pH 9.5 (EC-Apparatus Corp., technical bulletin no. 134). In the case of MDH, the buffer employed was tris-Na₂ EDTA-boric acid, prepared as described by Peacock et al. ('65), except that in the present experiments, the pH was adjusted to 8.0 (from pH 8.4) with additional amounts of boric acid.

The electrophoresis of LDH was performed at 4°C for three hours using a current of 400 v and 180 ma. In the case of MDH, the current was run for two hours 30 minutes using 300 v and 150 ma. The gels were stained for LDH by the method of Brewer ('70) and for MDH by the procedure of Weitkamp et al. ('69).

RESULTS AND DISCUSSION

A. Quantitative studies of heterokaryocyte formation

In order to measure the degree of heterokaryocytosis obtained in a given experiment, a parameter has been chosen which I shall call the *heterokaryocytic index*. It is defined as the proportion of nuclei, among all nuclei observed, which are present in heterokaryocytes. The heterokaryocytic in-

dex is therefore computed by dividing the number of nuclei in heterokaryocytes by the number of nuclei in all cells observed.⁴ If one assumes that, on the average, there is the same amount of cytoplasm associated with those nuclei which participate in cell fusion as with those which do not, then the heterokaryocytic index estimates the proportion of the total cytoplasm in the culture which is present in heterokaryocytes. Figure 1 shows a heterokaryocyte formed by the fusion of labelled CR cells with unlabelled MIS cells, both human diploid fibroblasts.

When mouse or Chinese hamster cells were exposed to tritiated thymidine under the conditions described earlier, 96 and

⁴ Estimation formula for the standard error of the heterokaryocytic index has proven very difficult to derive and is not yet available.

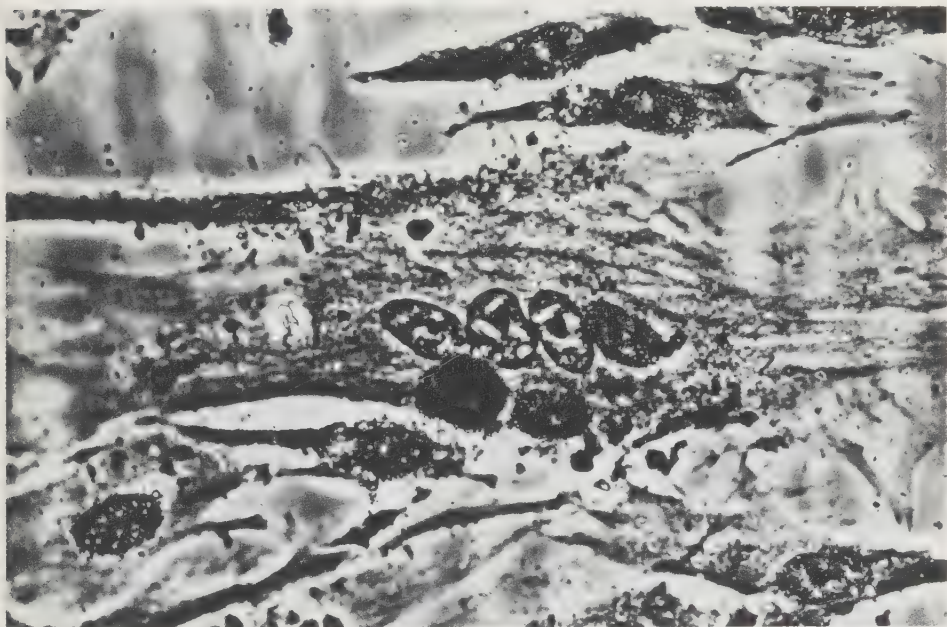


Fig. 1 An example of a heterokaryocyte formed by fusion of two lines of human diploid fibroblasts. Male cells (CR line) were labelled with H^3 -thymidine (labelling efficiency: 78.6%) and were mixed in a 1:1 ratio with unlabelled female cells (MIS line). Afterwards, the procedure described under MATERIALS AND METHODS was followed. The coverslips were then fixed, washed with cold TCA and autoradiographed. The heterokaryocyte shown in this picture contains three labelled and three unlabelled nuclei. Phase contrast microscopy. Magnification is approximately $\times 270$.

100% of their nuclei, respectively, became labelled. On the other hand, only 65 to 90% of the nuclei of human diploid fibroblasts incorporated the radioactive precursor to a measurable extent.⁵ A statistical method for estimating the heterokaryocytic index when the labelling of one of the parental lines with tritiated thymidine is incomplete has been described elsewhere (Velázquez, '70).

Figure 2 summarizes the results of several fusion experiments. When different strains of human diploid fibroblasts were fused with one another, the heterokaryocytic index was 0.238 in the first experiment (a), and 0.361 in the second (b). These figures suggest that roughly between 25 and 35% of the cells in mixed cultures of human diploid fibroblasts are incorporated into heterokaryocytes when exposed to Sendai virus under the present conditions. However, it should be added that this inference can rigorously be drawn only on the following assumptions: (1) no multinucleate cells arise by nuclear division;

(2) no cells or nuclei undergo division after the virus is added; and (3) no nuclear fusion takes place. These assumptions have been discussed in more detail by Velázquez et al. ('71).

While these experiments were in progress, Siniscalco et al. ('69) reported on the formation of hybrid cells between human diploid fibroblasts derived from two different donors. These authors presented evidence for the formation of heterokaryocytes between the two cell lines. However, they did not present data bearing on the degree of heterokaryocytosis obtained.

There is an obvious analogy between mammalian heterokaryocytes and the heterokaryons of higher fungi. The fungal heterokaryons, however, will grow and bear spores, whereas human heterokaryocytes

⁵ As human fibroblastic homonuclear cells age *in vitro* (Hayflick and Moorhead, '61; Hayflick, '65), the proportion of cells which engage in DNA synthesis, during a 24-hour interval, decreases (Macleira-Coelho et al., '66; Macleira-Coelho and Ponten, '69). Merz and Ross ('69) have shown that, over a period of months, as human homonuclear cells grow *in vitro*, the percentage of cells capable of undergoing division decreases exponentially.

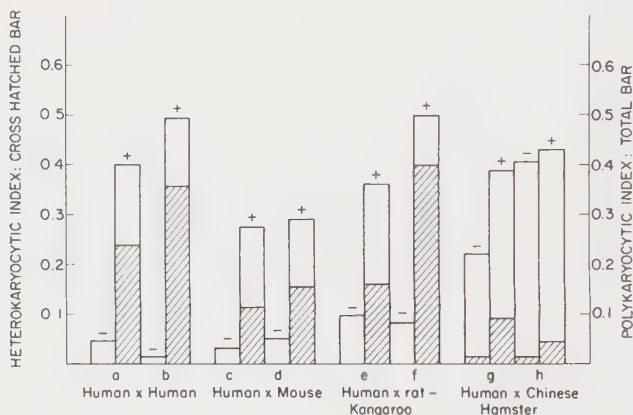


Fig. 2 Summary of the experiments measuring the degree of fusion of human diploid fibroblasts with other human cell strains and with cells from other species. The total area included in each bar represents the polykaryocytic index. The area included in the cross hatched segment of a bar represents the heterokaryocytic index. (—): cultures exposed to BSS; (+): cultures exposed to Sendai virus. (a) Labelled human cells, CR, mixed with unlabelled human cells, MIS. (b) Labelled human cells, JDU, mixed with unlabelled human cells, CR. (c) Labelled human cells, CR, mixed with unlabelled mouse cells, 3T3. (d) Labelled mouse cells, 3T3 mixed with unlabelled human cells, CR. (e,f) Two different experiments, having mixed labelled human cells, CR, with unlabelled rat-kangaroo cells, ROO. (g) Labelled human cells, CR, mixed with unlabelled Chinese hamster cells, CHA₂. (h) Labelled Chinese hamster cells, CHA₂, mixed with unlabelled human cells, CR.

do not grow. Hence, it is important to maximize the frequency with which human heterokaryocytes are formed. They cannot, unfortunately, be selected by devising media which would favor their overgrowth. However, a heterokaryocytic index for human-human mixtures, of 25 to 35%, which was obtained in the present work, is quite appreciable and may enable us to study complementation between different human genes. It is of interest to contrast heterokaryocytosis with somatic cell hybridization. This latter process occurs at frequencies lower than 1%, even after the addition of Sendai virus (Coon and Weiss, '69; Kao et al., '69).

The heterokaryocytic index observed after fusing human and mouse cells varies between 0.10 and 0.15 (fig. 2c,d). The heterokaryocytic index observed when human and rat-kangaroo cells were fused, was very different in the two experiments performed (fig. 2e,f); the index was 0.161 in the first experiment, and 0.398 in the second. The reason for this variation is unknown. In both of the above experiments, incidentally, the human nuclei were the ones labelled with tritiated thymidine. Experiments in which the rat-kangaroo cells were labelled suggest that the cultured cells of this animal are very sensitive to tritiated thymidine; after the cells are labelled, they do not survive throughout the fusion experiment. The Chinese hamster line used in these experiments normally contains 20 to 30% multinucleated cells. This is one of the reasons why the *polykaryocytic* index of the control human-hamster cell cultures is high.

As shown in figure 2, the mere co-cultivation of human and Chinese hamster cells seems to induce the formation of a small proportion of heterokaryocytes, which is further increased, though not to a high level, when Sendai virus is added. Although the polykaryocytic indices of mixed human-Chinese hamster cultures exposed to Sendai virus were similar in both experiments, the heterokaryocytic index varied over a two-fold range in these two experiments. I am presently unable to explain this difference. It should also be pointed out that the frequency of CR *homokaryocytes* seems to increase when cells of this line are co-

TABLE 1

Frequency of CR multinucleate cells (homokaryocytes) relative to all CR cells observed in pure cultures and in mixed cultures containing CR and CHA₂ cells. No Sendai virus added

	Pure CR cultures	Mixed CR + CHA ₂ cultures
Experiment 1	3.0	5.1
Experiment 2	4.8	13.0

cultivated with CHA₂ cells in the absence of virus (table 1).

Perhaps the fusion which takes place between human and hamster cells (and between different human cells co-cultivated with Chinese hamster cells) in the absence of added Sendai virus, might be due to a virus carried by the Chinese hamster cells — a virus whose presence is covert. This idea is supported by electron microscopic observations which show that many lines of hamster cells contain virus-like particles (Bernhard and Tournier, '64; Compans et al., '66). Recently, Jensen et al. ('70) have reported the isolation of a paramyxo-like virus from a line of SV-40 transformed hamster cells. They found that this virus induces the formation of polykaryocytes in cultures of other lines of hamster cells.

B. Studies on the electrophoretic mobility of the lactate and malate dehydrogenase activities recovered from mixed human-mouse cultures following exposure of the cultures to Sendai virus

Two experiments were performed in order to study the electrophoretic mobility of the LDH and MDH activity recovered from mixed human-mouse cultures. Cells were added to flasks at appropriate density to form a confluent monolayer. One set of flasks received a mixture of CR and 3T3 cells in a 1:1 ratio. Two other sets contained pure cultures of CR or 3T3 cells. Each set was subdivided into two groups, one which received the virus, the other receiving BSS instead. In the first experiment, the cells were harvested one day after addition of the virus. In the second experiment, the cells were harvested on the ninth day. The polykaryocytic indices, for these two experiments were 0.311 and 0.296, respectively. It was decided not to label the nuclei of one of the cell lines in

order to estimate the heterokaryocytic index, because H^3 -thymidine may damage or kill cells which incorporate it (Drew and Painter, '59), and thus interfere with studies on the cellular protein synthesis. Furthermore, the electrophoretic experiments required large quantities of cells, and labeling one of the parental lines would be very costly.

In both experiments, the electrophoretic patterns of mixed cultures exposed to Sendai virus were indistinguishable from the control ones (mixed but without virus). The patterns of the virus-exposed cultures were also indistinguishable from those produced by a 1 : 1 mixture of cell extracts from control cultures of both parental lines. As shown in figures 3 and 4, no additional bands were detected. On the other hand, new LDH bands, not observed in extracts from the parental cultures, were observed in extracts of cells from a human-mouse hybrid clone (MIG). These new bands very likely correspond to hybrid isozymes (fig. 3). No hybrid MDH bands were observed in extracts from MIG cells (fig. 4). This could well be due to the loss of human chromosomes containing the locus for MDH in this clone, since most human chromosomes are eventually lost in human-mouse hybrids (Weiss and Green, '67).

The absence of hybrid enzymes in cultures containing heterokaryocytes was unexpected, since hybrid isozymes of LDH and MDH have been observed in clones of human-mouse hybrid lines (fig. 3; Nabholz et al., '69; Boone and Ruddle, '69). Moreover, there is evidence that, at least for some loci, genes of both nuclei are expressed in mammalian heterokaryocytes. In Hela-Ehrlich ascitis tumor cell heterokaryocytes, antigens of both man and mouse are present on the cell surfaces, and seem to be randomly mixed (Watkins and Grace, '67).

In the present study some of the cultures, at the time of harvest, had been exposed to virus as long as nine days earlier. One would think this interval provided ample time for the cytoplasm of the heterokaryocytes to become thoroughly mixed.

There is the possibility that the heterokaryocytes did not synthesize protein and that the protein present in the parental cells at the time of cell fusion was not

replaced by *de novo* synthesized protein and hence no hybrid enzymes were formed. An experiment was therefore performed in order to determine whether or not the cultures containing human-mouse heterokaryocytes are able to engage in protein synthesis. In cultures containing a 1 : 1 mixture of human (CR) and mouse (3T3) cells exposed to Sendai virus, the ability of all cells, both mono and polykaryocytes (which presumably include the heterokaryocytes) to incorporate tritiated leucine into their respective acid-insoluble pools was measured. In this experiment, the polykaryocytic index of the cultures exposed to virus was 0.258.

At days 1, 4 and 7 after addition of the virus, the cells were subcultured at low cell population density into dishes containing coverslips and nucleomedium. Fifteen hours later, the cells were washed twice in leucine-free automedium and labelled with tritiated leucine for two hours. Two sets of dishes (each set containing control and virus-treated cultures) were exposed to tritiated leucine in automedium containing no cold leucine, one of these sets also containing cycloheximide (Acti-dione, Nutritional Biochemical Corp., Cleveland, Ohio) at a final concentration of 100 μg per milliliter. A third set of cultures was exposed to tritiated leucine in complete automedium, which contained about 500 times more cold than radioactive leucine. Afterwards they were washed twice, fed with nucleomedium and incubated at 37°C for 45 minutes. The coverslips were then washed, fixed and submerged in 0.3 N trichloroacetic acid at 4°C for one hour. The coverslips were washed in distilled water and 70% ethanol before being mounted and studied autoradiographically. In all the cultures to which H^3 -leucine was added to leucine-free automedium, all the cells, both mono and polykaryocytes, became labelled. It seems likely that most of the radioactivity was indeed in leucine at the time of being incorporated, because cultures in complete automedium rather than in leucine-less automedium did not become labelled. The incorporation of tritiated leucine to the acid insoluble pool of the cells is almost certainly a consequence of protein synthesis. No cell was labelled when

LDH Electrophoretic Patterns

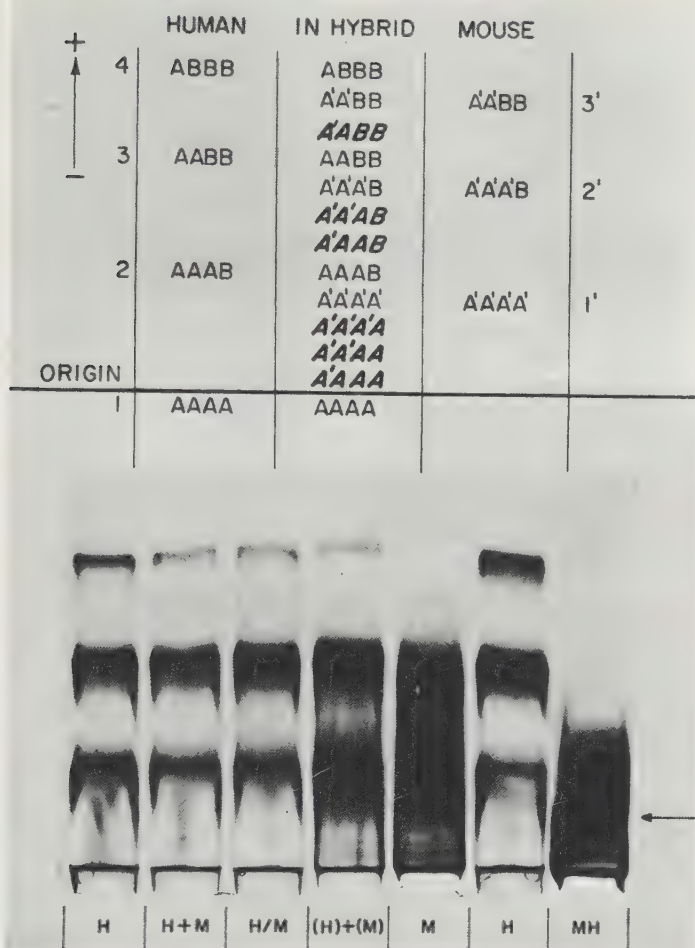


Fig. 3 LDH electrophoretic patterns of extracts from human, mouse and human-mouse cell cultures. Upper half: Diagrammatic representation of the mobility and composition of LDH isozymes from pure and hybrid human-mouse cultures in acrylamide gels (from Nabholz et al., '69; Boone and Ruddle, '69). B human and mouse subunits have the same mobility; A human subunit moves slower than A' mouse subunit. Theoretically distinguishable hybrid isozymes are represented by bold-face letters. Lower half: Observed patterns of extracts prepared as described under MATERIALS AND METHODS, and electrophoresed in acrylamide gel. Cells were harvested after nine days of exposure to BSS or virus. H: extracts from human cells; M: extract from mouse cells; H + M: extract from mixed culture of human and mouse cells exposed to BSS; H/M: extract from mixed human-mouse culture exposed to virus; (H) + (M): 1:1 mixture of human and mouse cell extracts; MH: extract from a clone of human-mouse hybrid cells. Putative hybrid bands are designated by the arrow in the lower part of the figure.

MDH Electrophoretic Patterns

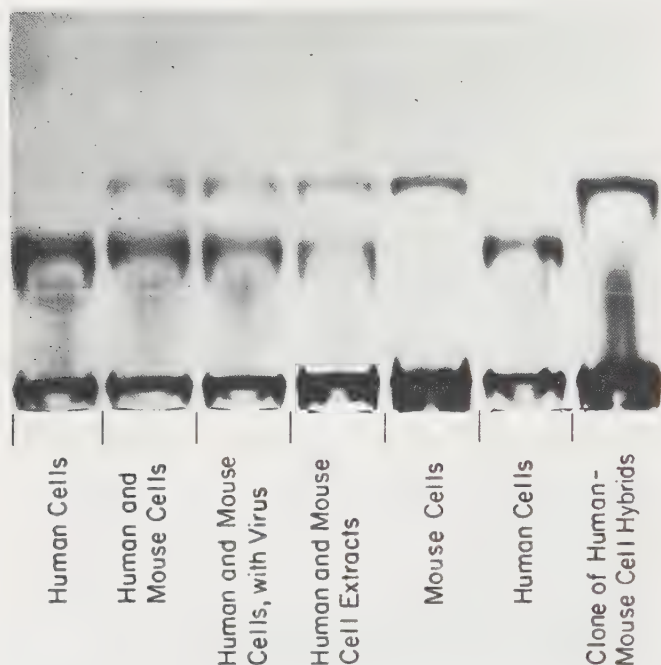


Fig. 4 MDH electrophoretic patterns of extracts from human, mouse and human-mouse cell cultures. Cells were harvested after nine days of exposure to BSS or virus, and extracts were prepared as described under MATERIALS AND METHODS.

the pulse was administered in the presence of cycloheximide, an inhibitor of protein synthesis, which appears to act by blocking the formation of peptide bonds (Wettstein et al., '64; Colombo et al., '65). Therefore, human-mouse heterokaryocytes appear capable of synthesizing protein, at least as far as can be determined from the behavior of the polykaryocytes recovered from mixed cultures. Moreover, this synthesis can be demonstrated for at least seven days after the culture has been exposed to virus. Harris et al. ('66) have also demonstrated that heterokaryocytes formed by human cells and cells derived from other species, including fowl, are capable of incorporating tritiated leucine.

There are two alternative possibilities which might explain the absence of hybrid isozymes in virus-treated mixed cultures, in spite of their apparent capacity for continued protein synthesis. The genes of each nucleus may retain the control of their corresponding cytoplasm, so that polypeptide subunits coded by genes in different nuclei will not form part of the same molecule. Alternatively, hybrid enzymes may indeed be formed, but not in detectable amounts, due to the relatively low frequency of heterokaryocytes. Some support for the first possibility comes from an observation by Siniscalco et al. ('69). These authors found that even 48 hours after virus exposure a mixed culture of normal and deficient cells

for glucose-6-phosphate dehydrogenase contained binucleated cells in which the enzyme could be detected histochemically around only *one* of the two nuclei. Pontecorvo ('52) has suggested that genes combined at different levels of cellular organization may exhibit different phenotypes. For example, in *Didymium iridis* complementation between two non-allelic pigment mutants occurs in the heterozygous state, but not in diploid heterokaryons (Collins, '69).

On the other hand, it is possible that hybrid enzymes are indeed formed in human-mouse heterokaryocytes, but because the heterokaryocytic index of the cultures is not sufficiently high their concentration in the extract is below the limits of detection. A possible means of deciding between these two possibilities might be by fractionating the cell population on the basis of cell size, employing sedimentation velocity techniques (Miller and Philips, '69), and in this way obtaining a fraction rich in heterokaryocytes.

ACKNOWLEDGMENTS

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Selective Gene Expression in All Hybrids

IMMUNOCHEMICAL DETECTION OF A HUMAN SPECIES-SPECIFIC ESTERASE IN INTERSPECIES HYBRID CELLS

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BARBARA B. KNOWLES and N. R. ROSE

An esterase isoenzyme peculiar to human tissues has been described previously [1]. This esterase is identified as the most cathodal of the human tissue esterases, migrating in the γ globulin region during electrophoresis in agarose at pH 8.2. Rabbit antisera containing antibodies against this esterase isoenzyme provided a sensitive and specific tool for its detection in various tissues and cell lines. Enzyme-immunoelectrophoresis and gel diffusion techniques using the anti-esterase sera have shown this antigen to be present in human and Rhesus monkey tissues, but not in the tissues of the other species tested, including mouse, rat, hog, guinea pig, beef, goat, and chicken [1, 2].

With the availability of somatic hybrid cell lines containing the combined chromosomal complement of two different species, human-mouse or monkey-mouse, we had at our disposal a system for the utilization of this esterase as a specific human or monkey genetic marker. The hybridization of cells from human and mouse or monkey and mouse cell lines results

in the subsequent gradual loss of human and monkey chromosomes, while the complement of mouse chromosomes remains almost unaltered [3]. Since the esterase under study was present in human and monkey somatic cells, but not in those of the mouse, it was intriguing to look for somatic cell hybrids in which the primate chromosome(s) carrying the gene coding for this esterase might be retained.

Therefore, a number of human-mouse and monkey-mouse hybrid clones were tested immunochemically for the presence of the human esterase isoenzyme in an attempt to correlate its presence or absence with the primate contribution to the karyotype of these cells.

MATERIALS AND METHODS

Cell lines

Hybrid cells were produced by fusion with β -propiolactone-inactivated Sendai virus and chemical selection [4]. The human fibroblast line K1 was derived from a patient with Lesch-Nyhan syndrome, and hence lacked the enzyme hypoxanthine-guanine phosphoribosyl transferase. Cl-1-D cells were of murine origin,

derived from an L cell lacking thymidine kinase. Fusion between these parent lines and growth in selective medium containing aminopterin produced the hybrid cell, 133-5. Two clones from this experiment were studied, 133-5-cl-1 and 133-5-cl-2. Another hybridization between the same parental lines gave 102-2, of which three clones were studied, 102-2-cl-3, 102-2-cl-4, and 102-2-cl-7.

The human-mouse hybrid, HM₂P₃ (obtained from Dr Mary Weiss), was derived from the fusion of cl-1-D with the human diploid line WI-38. Human peripheral blood leucocytes were fused with IT, thymidine kinase deficient mouse cells, and two clones of this 243 series of hybrids (243-3A and 243-1B) were investigated. The 193 clone was derived from a hybridization of EB-2 cells (Burkitt lymphoma line) and MKS BU-100 (SV-40 transformed mouse line). The "P" clone (provided by Dr V. Miggiaro) was derived from the fusion of African green monkey peripheral blood cells with the mouse line, IR.

In each of these fusions the lymphoid parent was eliminated by extensive washing and the glass-attached drug resistant parent was eliminated by growth in medium containing aminopterin. Hybrid colonies were then removed from the Petri dish and single cell clones derived from them were used for further study.

Parent cells were grown on 2X basal medium of Eagle, (BME, GIBCO) containing 10-20% fetal bovine serum and 50 µg/ml chlortetracycline. Drug-resistant parental lines cl-1-D, MKS BU-100 and IT were grown in the presence of 100 µg/ml 5-bromodeoxyuridine. Hybrid cells were cultured in GHAT medium (3×10^{-6} M glycine, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 6.6×10^{-8} M thymidine) containing 10-20% fetal bovine serum and chlortetracycline, in order to eliminate the drug-resistant parental cells from the population [5].

Cell extracts

Extracts were prepared from parent and hybrid cell lines propagated in Blake bottles. Approx. 20 bottles were used to obtain a cell pellet having a wet weight of 0.5 to 0.7 g. The method used for harvesting and preparing extracts of these cells has been described in a previous publication [1]. Extracts of these cell lines usually yielded protein concentrations (detected by biuret) of 10 to 30 mg/ml.

Chromosome analysis

Samples of cells were selected coincident with their harvesting for enzymatic studies and the chromosome patterns analysed [6]. The approximate number of primate chromosomes was estimated from the number of banded chromosomes in excess of those found in the parental mouse cells.

Immunological and histochemical techniques

Immunoelectrophoresis and double diffusion in agarose, as well as the histochemical staining techniques for esterase, have been described previously [1]. Extremely small quantities of cell extract (less

than 10 λ) were required to fill each antigen well for enzyme-immunoelectrophoretic analysis.

Antisera against the human cathodal esterase were prepared in a number of rabbits. Whole cells of WI-38, a human diploid line, were injected into rabbits. After at least six to eight monthly intradermal injections with complete Freund adjuvant, antibodies appeared to a number of cellular constituents. One of these antibodies was found to be directed against the "cathodal" esterase of WI-38 [1].

One antiserum reacted with the cell extracts to give only one precipitation arc which was enzymatically active. By combining immunoelectrophoresis or double diffusion with the esterase assay, this antiserum could be used as a specific reagent for the detection of the human cathodal esterase—the one antigen with esterase activity.

Comparative experiments using this rabbit antiserum demonstrated that the WI-38 esterase was immunologically identical with the most cathodally migrating esterase in human and monkey tissues. The species-specificity of the antiserum was demonstrated by its reaction with the cathodal esterase of human and African green monkey cells, and its failure to react with the tissue and cell extracts of other species [1, 2].

RESULTS

Enzyme-immunoelectrophoresis was performed using rabbit antiserum against WI-38 to detect the human cathodal esterase. The human lines, K1 and WI-38, possessed the cathodal esterase, while the mouse lines, cl-1-D, IT, and IR did not. Of all the clones tested from the cross K1 $\times$ cl-1-D, only one, 133-5-cl-1, exhibited the esterase (fig. 1). Since this clone was found to be extremely polyploid, adequate chromosome analysis could not be performed. Hybrids 102-2-clones 3, 4 and 7 contained, respectively, 5, 8, and 12 more banded chromosomes than did the cl-1-D mouse parent. The 133-5-cl-2 contained only one extra banded chromosome over the cl-1-D background.

The double diffusion method of Ouchterlony, a more sensitive technique than immunoelectrophoresis, confirmed these results. Again 133-5-cl-1 was the only hybrid culture demonstrating esterase activity with specific antiserum. The esterase isoenzyme of the hybrid was shown to form a line of identity

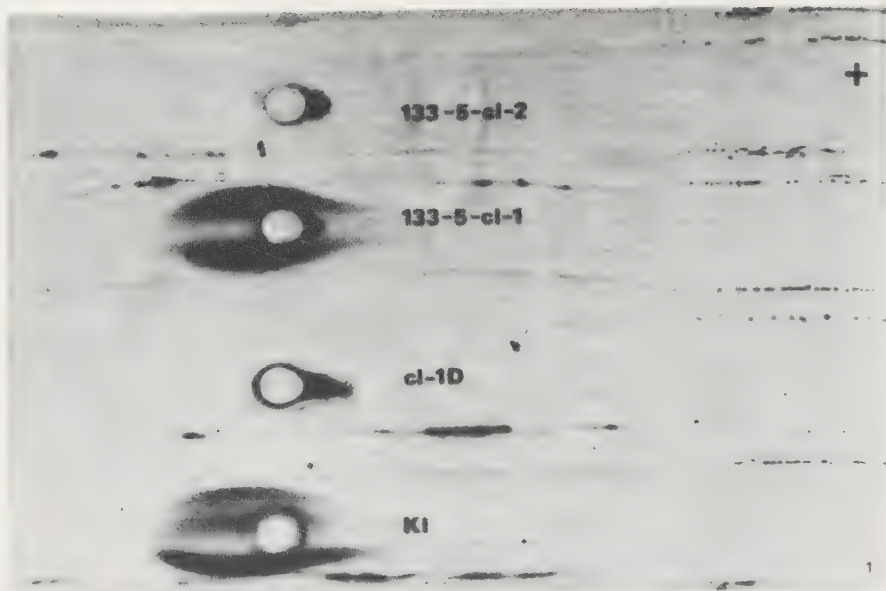


Fig. 1. Esterase detected by enzyme-immunoelectrophoresis in extracts of human parent, KI and hybrid, 133-5-cl-1 by anti-WI-38 rabbit serum. Esterase was absent from extracts of murine parent, cl-1-D and hybrid, 133-5-cl-2.

with WI-38 (fig. 2). HM_2P_5 , a hybrid line containing three extra biarmed chromosomes at the time of testing, was esterase negative. Clones of two other human-mouse hybrids, 243 and 193, were examined by double diffusion and found to lack the characteristic esterase. At the time of testing, the 243 clones contained between one and five biarmed chromosomes, presumably of human origin, and the 193 clone contained nine chromosomes morphologically similar to human chromosomes.

Similar experiments utilizing the techniques of enzyme-immunoelectrophoresis and enzyme-immunodiffusion were undertaken to examine the monkey-mouse hybrid clone, 2P. Esterase activity was detectable in 2P throughout its period of propagation in our laboratory. Although the identification of the species origin of the chromosomes was

not practical in this cross (IR mouse parent contained 18 biarmed chromosomes), there was an excess of 14 biarmed chromosomes over the background IR number, and additionally 10 more telocentric chromosomes than in the IR parent.

DISCUSSION

Certain investigators have lamented the scarcity of phenotypic genetic markers in somatic cells [7, 8]. Recently, the use of isoenzymes for this purpose was exploited by Ruddle et al. [7]. In comparing the presence or absence of variants of a wide number of enzymes in human-mouse hybrid cells, they were able to propose linkage groups for certain enzymes, and to assign them tentatively to specific human chromosomes.

Electrophoresis followed by staining for

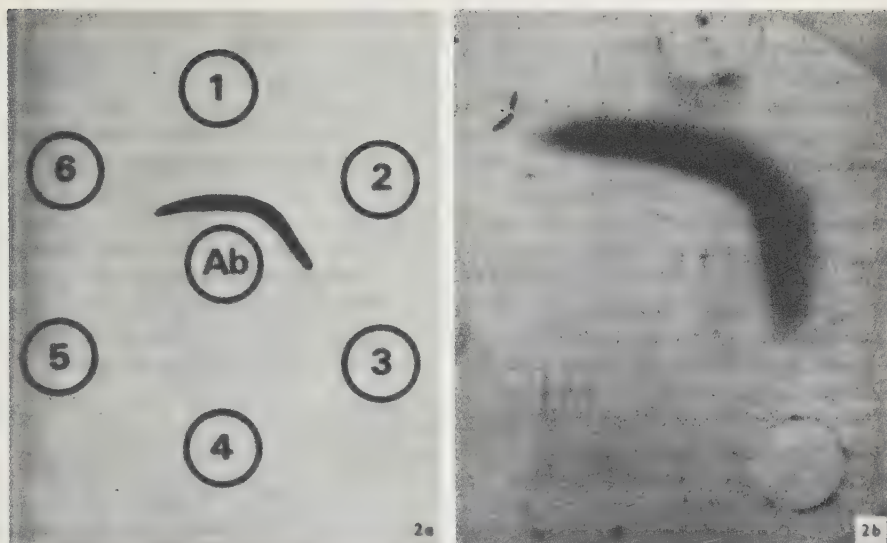


Fig. 2. (a, b) Ouchterlony test showing immunological identity between WI-38 and the hybrid, 133-5-cl-1, extracts employing an antiserum against WI-38. All the other hybrid cells tested here lacked the esterase. Well 1, WI-38; 2, 133-5-cl-1; 3, cl-1-D; 4, 133-5-cl-2; 5, 102-2-cl-3; 6, 102-2-cl-4; Ab, rabbit antiserum to WI-38.

enzyme activity, as a broad-scale screening method for determining the presence or absence of specific isoenzymes in cell or tissue extracts, is useful. However, the utility of this method is limited by its relative lack of sensitivity and specificity. At times, a certain isoenzyme may be overlooked because its concentration in a particular extract is below the point of resolution. Also, isoenzymes of various cell extracts may possess the same electrophoretic mobility, but possible antigenic differences may be undetected by this method.

Inherently more specific and more sensitive than electrophoresis are the immunological techniques of immunoelectrophoresis and double diffusion. The increase in sensitivity of the immunological techniques applied to enzyme analysis is accomplished by the concentration of enzyme in an antibody-enzyme

lattice which forms as a precipitation arc in a limited area in the agarose matrix. An example of this added sensitivity is illustrated in our former study [1] in which transformed WI-38 cell extracts, assayed by electrophoresis for esterase activity, were found to be devoid of cathodal esterase. When the same extracts were subjected to immunoelectrophoresis with antiserum against the cathodal esterase, no precipitation arc was seen. However, when the esterase staining procedure was carried out, an esterase arc was seen in the cathodal region, which indicated that at least a trace of this isoenzyme was present in the transformed cells. Although both extracts were initially adjusted to contain the same protein concentration, titrations of the two extracts (WI-38 and the transformed WI-38) revealed a 5-fold reduction in cathodal esterase in transformed cells. Enzyme-immunoelectro-

phoresis has thus proved to be more sensitive than electrophoresis or an immunoelectrophoretic test utilizing protein stains.

The specificity of immunological reactions provides a method of discriminating among enzymes of similar electrophoretic mobility. For instance, it has been our experience to find esterases of guinea pig and bovine tissues with electrophoretic mobilities identical to the cathodal esterase of human tissues. However, using an antiserum against the human cathodal esterase, it was possible immunologically to distinguish the human cathodal esterase from the esterases of other species [2].

The human cathodal esterase isoenzyme under study in our laboratory is one electrophoretic enzyme variant which has proven useful as an immunologically defined genetic marker for the presence of a certain human or monkey chromosome(s) in cells hybridized from either of these species. By employing antisera against WI-38 in immunoelectrophoresis and double diffusion in gel, and combining these methods with a selective staining technique, the cathodal esterase could be detected consistently in all cell lines of exclusively human or monkey origin which we have studied. It was also present in the human and monkey parent cells used for hybridization.

Since this esterase has been detected in only two of the ten hybrid lines studied, it seems that the primate chromosome that

carries the gene governing the expression of the esterase isoenzyme is frequently lost after hybridization with mouse cells, or is at least not selectively maintained. These results further indicate that this human species-specific antigen can be expressed in a cell containing predominantly mouse chromosomes. The possibility of genetic or epigenetic repression of the human cathodal esterase by the mouse parent (such as that reported by Klebe et al. [9] for mouse ES-2) seems unlikely, as the mouse chromosomes appear to remain constant in our hybrid lines.

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FURTHER STUDIES ON THE INDUCTION OF ALKALINE PHOSPHATASE BY 5-BROMODEOXYURIDINE IN A HYBRID LINE BETWEEN MOUSE AND CHINESE HAMSTER IN CULTURE

HIDEKI KOYAMA AND TETSUO ONO

INTRODUCTION

In the preceding paper¹, we gave a preliminary report of the induction of alkaline phosphatase activity by 5-bromodeoxyuridine in one hybrid line, designated B-6, derived from somatic cell hybridization of mouse and Chinese hamster cell lines *in vitro*². This line has the inheritable ability to produce hyaluronic acid³, and this function is reversibly inhibited by treatment with the analog⁴. At that time, the activity of hyaluronic acid synthetase responsible for the final step of its biosynthesis was found to be reduced significantly in a cell-free extract of the analog-treated cultures, in contrast with alkaline phosphatase.

In the present study, we characterized in more detail the induction process

of alkaline phosphatase in B-6 cells, and then compared the two inverse actions of 5-bromodeoxyuridine: inhibition of hyaluronic acid synthesis and induction of alkaline phosphatase activity. As a result, both reactions were found to correlate closely with each other, strongly suggesting that the same or common causal mechanisms operate in the cells exposed to the analog, not only to suppress the mucopolysaccharide production but also to elevate the alkaline phosphatase activity. Therefore, studies on the mechanism of the phosphatase induction in this system is considered to contribute to the understanding of the inhibitory actions of 5-bromodeoxyuridine on differentiated functions⁵⁻¹⁰ as well as on hyaluronic acid synthesis, or on the tumorigenicity of mouse melanoma cells¹¹.

MATERIALS AND METHODS

Cells and culture methods

The origin and properties of the B-6 line used throughout the present study were described previously²⁻⁴. Cells were grown as floating cultures in 100-mm glass dishes containing a standard medium in a humidified atmosphere of CO₂-air (5:95, v/v), and subcultured twice a week by transferring them to fresh medium with appropriate dilution. The medium consisted of a modified Eagle's minimum essential medium¹² (Nissui Seiyaku, Tokyo), 5 % inactivated calf serum and 0.1 % Bacto-Peptone (Difco, Detroit).

Treatment of cells with 5-bromodeoxyuridine or other reagents

Actively growing cells were collected by low-speed centrifugation, and cell count was performed in a Thoma-type hemocytometer by means of dye exclusion test using 0.25 % trypan blue solution. The viable cells were inoculated at a concentration of $5 \cdot 10^5$ cells per dish into 100-mm replicate glass dishes, each containing 10 ml of the standard medium with 5-bromodeoxyuridine or other reagents, except for the growth experiment shown in Fig. 1, which was performed by seeding $2.5 \cdot 10^5$ cells on 50-mm Toyoshima plastic dishes (Toyoshima Seisakusho, Tokyo) in 5 ml of the same medium with 5-bromodeoxyuridine. These cultures were incubated in the CO₂ incubator kept in the dark for various lengths of time at 37 °C. In the experiments to examine the effect of 5-bromodeoxyuridine on growth curve and viability, a part or whole of duplicate cultures, depending on the cultural days after start of experiments, was packed by low-speed centrifugation in small tubes, and the number of viable and total cells were counted after staining in the trypan blue solution. In other enzyme experiments, a portion (0.5-1 ml) of duplicate cultures was stocked as pellets at -70 °C to estimate cell growth by a chemical method (protein content), and the remaining to assay enzyme activity.

Assay of alkaline and acid phosphatase activity

Cell-free enzyme preparations were made by washing three times the thawed cell stocks with ice-cold saline, followed by suspension into 0.5-2.0 ml of 0.25 M sucrose and then sonication using a Quigley-Rochester 10 KC oscillator with cooling in ice-water. The alkaline phosphatase activity of these samples was measured by the hydrolysis of *p*-nitrophenyl phosphate (8 mM) as substrate in 0.5 M 2-amino-2-methyl-1-propanol buffer at pH 10.0 according to the method of Lowry¹³. Reaction

mixtures consisting of 0.5 ml of the substrate-buffer solution plus 0.1 ml of enzyme solution (50–200 μg protein) were incubated for 30 min at 37 °C. Then the reaction was stopped by the addition of 1.5 ml of 0.25 M NaOH and the amount of *p*-nitrophenol liberated was measured at 410 nm in a Beckmann DB spectrophotometer. The activity of acid phosphatase was assayed under the same conditions except 0.1 M sodium acetate buffer, pH 4.8, was used in place of the above alkaline buffer and each assay tube contained 5–30 μg protein.

The enzyme activity (specific activity) was expressed as nmoles of *p*-nitrophenol released in 1 h per mg protein. Protein contents in enzyme solutions and in whole cells for the assay of cell multiplication were determined by the method of Lowry *et al.*¹⁴, as modified by Oyama and Eagle¹⁵, using bovine serum albumin as a standard.

Chemicals

5-Bromodeoxyuridine, 5-bromodeoxycytidine, 5-bromouracil, 5-bromouridine and thymidine were obtained from Sigma Chem. Co., St. Louis. 5-Iododeoxyuridine was purchased from Kojin Co., Tokyo, and disodium *p*-nitrophenolphosphate from Daiichi Pure Chem., Tokyo.

RESULTS

Effect of 5-bromodeoxyuridine on growth curve and viability

We have studied previously the effect of a 3-day exposure to 5-bromodeoxyuridine on the growth of the B-6 line and found the presence of some inhibitory action at 5 $\mu\text{g}/\text{ml}$ or more⁴. In the present study, this was reexamined in more detail with reference to the effect on the growth curve and viability of the cells, as shown in Fig. 1. During the first 48 h after exposure to the analog, there was little or no

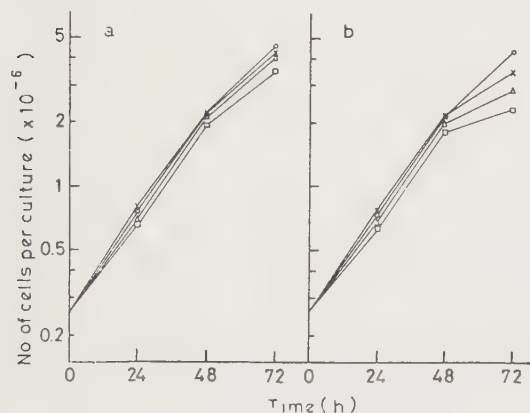


Fig. 1. Effect of various concentrations of 5-bromodeoxyuridine on the growth curve and viability of B-6 cells during 3 days of incubation. The assay method was described in Materials and Methods, and the numbers of total and viable cells per culture are plotted against incubation time in (a) and (b), respectively. The concentrations of 5-bromodeoxyuridine added ($\mu\text{g}/\text{ml}$) are: $\circ$ — $\circ$, zero; $\triangle$ — $\triangle$, 1; $\square$ — $\square$, 2.5; $\diamond$ — $\diamond$, 5; and $\times$ — $\times$, 25.

effect on both cell growth and viability at all concentrations (1–25 $\mu\text{g/ml}$) examined. The cells grew exponentially without lag, and the mean doubling time was 15.9 h, as calculated from the slopes of growth curves between the 1st and 2nd days. In this period, cell viability was more than 95 % in these 5-bromodeoxyuridine-treated cultures as well as in controls.

Subsequently, during the incubation time from 48–72 h, there was found a small decline of increase in the numbers of total and viable cells (Figs 1a and 1b, respectively) in both treated and untreated cultures. The decrease in their growth rates was in proportion to increasing concentrations of 5-bromodeoxyuridine added, and this effect was even more apparent for cell viability. After 3 days, the percent of viable cell number relative to the total number of cells was approximately 95, 92, 84 and 78 % in the cultures grown in the analog at 0, 1, 5 and 25 $\mu\text{g/ml}$, respectively.

The B-6 line was able to propagate in 5-bromodeoxyuridine for at least 18 days. Fig. 2 represents a cumulative increase in the total number of cells per culture during serial subcultivation in the presence or absence of the analog. Control cells always proliferated exponentially with a mean doubling time of 17.5 h. Also, in the presence of 1 $\mu\text{g/ml}$ of 5-bromodeoxyuridine, the cells could propagate at almost the same rate as untreated cells. The growth rate of cultures treated with 5 $\mu\text{g/ml}$, however, was reduced considerably after the second transfer generation. Thereafter, the

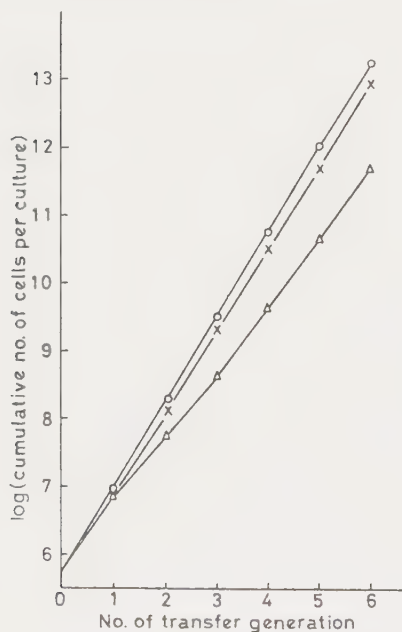


Fig. 2. Effect of a prolonged subcultivation on the growth of B-6 cells in medium with or without 5-bromodeoxyuridine. Cells at an inoculum size of $5 \cdot 10^3$ cells per 100-mm glass dish were serially subcultured every 3 days in medium with or without 5-bromodeoxyuridine and, at each time of transfer, the total number of cells and viability were determined by cell count. $\circ$ — $\circ$, control; $\times$ — $\times$, 1; and $\triangle$ — $\triangle$, 5 $\mu\text{g/ml}$ of 5-bromodeoxyuridine added. One transfer generation means 3 days.

increase in cell number occurred linearly throughout the 15-day period assayed, with a somewhat decreased mean generation time of 23.0 h. The viability of cultures treated continuously with the concentrations of 0, 1 and 5 $\mu\text{g/ml}$ was on average 98, 95 and 88 %, respectively.

From these results, it is evident that both the growth and viability of the B-6 line are suppressed to some degree, when grown with 5-bromodeoxyuridine at concentrations of 5 $\mu\text{g/ml}$ or more for over 48 h, but that this effect is not sufficient to result in complete death of the cells even upon a relatively prolonged cultivation in the analog.

Time course of alkaline phosphatase induction

Figs 3 and 4 show the time course of the induction of alkaline phosphatase activity by 5-bromodeoxyuridine in B-6 cells. In Fig. 3, the phosphatase activity in control cultures was little changed during the 72-h incubation, while a marked elevation in specific activity occurred in 5-bromodeoxyuridine-treated cultures.

During the first 24 h, the increase in the enzyme activity was only less than 50 % or sometimes was not detectable, but thereafter the induction began in the cells. In the cultures exposed to 5 $\mu\text{g/ml}$ of 5-bromodeoxyuridine, the rise in activity proceeded linearly, and did not level off, throughout the 72-h period. On the other

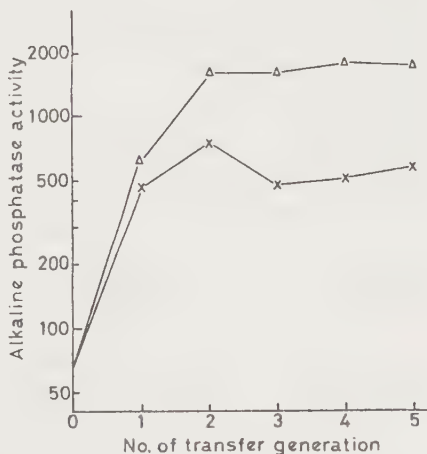
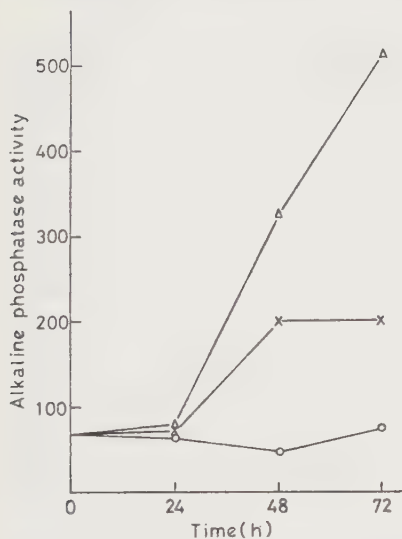


Fig. 3. Time course of alkaline phosphatase induction during 3-day growth of B-6 cells in 5-bromodeoxyuridine. The method for this experiment was described in Materials and Methods. The alkaline phosphatase activity is expressed as nmoles of *p*-nitrophenol liberated in 1 h per mg protein at 37 °C. The symbols are the same as those in Fig. 2.

Fig. 4. Fully-induced levels of alkaline phosphatase following a prolonged subcultivation of B-6 cells in 5-bromodeoxyuridine-containing medium. Cells were subcultured in the same manner as described in Fig. 2 and, at each time of transfer, the activity of alkaline phosphatase was assayed. $\times$ — $\times$, 1; and $\triangle$ — $\triangle$, 5 $\mu\text{g/ml}$ of 5-bromodeoxyuridine added continuously. One transfer generation means 3 days.

land, the cultures exposed to $1 \mu\text{g/ml}$ showed a 3-fold higher activity than did control cultures at 48 h, and during the time from 48–72 h there was no longer a rise in activity. This plateau level may be due to the exhaustion of the analog from the medium, due to cell proliferation during incubation for more than 48 h.

Fig. 4 illustrates the induction curves of alkaline phosphatase in the cells subcultured in 5-bromodeoxyuridine-containing medium for a longer time than 3 days in the same manner as described in Fig. 2. The activity continued to rise during the first 2 transfer generations (6 days) and reached its maximum within 6–9 days. This fully-induced level of activity was about 550 and 1700 nmoles/h per mg protein in the cultures treated with 1 and $5 \mu\text{g/ml}$, respectively, of the analog. The maximum level remained unchanged upon successive growth in 5-bromodeoxyuridine for at least 3 generations (9 days) after the 2nd generation.

Reversibility of alkaline phosphatase induction

When B-6 cells proliferated in 5-bromodeoxyuridine were transferred to the analog-free, fresh medium and subcultured every 3 days, the elevated phosphatase activity was observed to reduce again to the original value.

In Fig. 5, the activity which had elevated up to 5.5 times that of controls after 3 days of treatment with 5-bromodeoxyuridine (zero transfer generation)

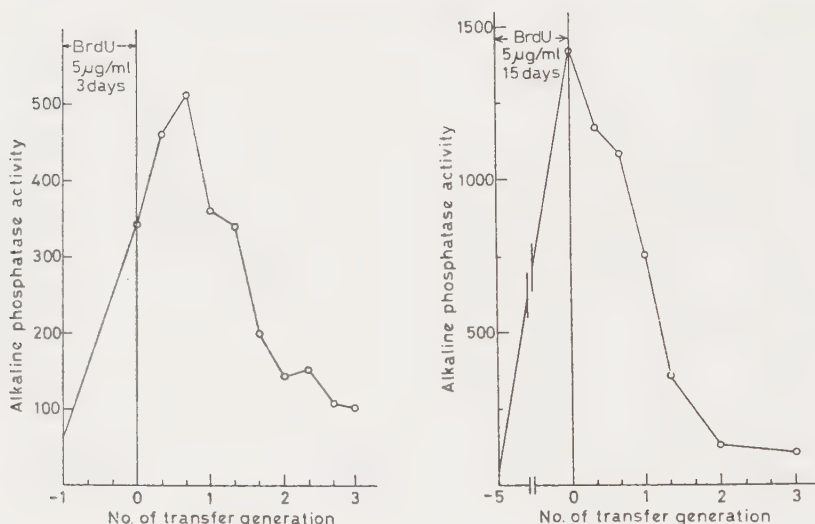


Fig. 5. Reduction curve of induced alkaline phosphatase activity in the B-6 cells grown in 5-bromodeoxyuridine for 3 days, following transfer into the analog-free medium. Cells were treated for 3 days with 5-bromodeoxyuridine ($5 \mu\text{g/ml}$). Then they were washed twice with the analog-free medium, transferred into the standard medium and subcultured every 3 days. The activity of alkaline phosphatase was assayed every day after zero transfer generation.

Fig. 6. Reduction curve of fully-induced alkaline phosphatase activity after successive exposure of B-6 cells to 5-bromodeoxyuridine for 15 days. The method for this experiment was the same as described in Fig. 5, except that B-6 cells were grown in the presence of 5-bromodeoxyuridine ($5 \mu\text{g/ml}$) for 5 generations or 15 days. One generation time means 3 days.

continued to rise further during 2 days after removal of 5-bromodeoxyuridine, and then decreased progressively to the basal level in 7 days.

In contrast, the reduction curve of higher alkaline phosphatase, which was obtained from the cultures returned to normal medium after prolonged incubation (15 days) of B-6 cells in the presence of the analog, differed from the above curve in two respects (Fig. 6). First, in the latter case, the decline in induced activity began on the next day following transfer of treated cells to normal medium, without any subsequent increase. Secondly, it took a shorter length of time (2 transfer generations; 6 days) in Fig. 6, as compared to 3 generations or 9 days in Fig. 5, until the induced activity returned to the basal level. In other experiments, the reversibility was not improved by addition of thymidine (20 $\mu\text{g}/\text{ml}$) to the cells after removal of 5-bromodeoxyuridine.

Suppression of alkaline phosphatase induction by simultaneous addition of thymidine with 5-bromodeoxyuridine

Inhibition of hyaluronic acid synthesis by 5-bromodeoxyuridine has been found to disappear when thymidine is added together with the analog to cultures⁴. Accordingly, we examined whether thymidine might block the induction of alkaline phosphatase by 5-bromodeoxyuridine. Table I summarizes the results. The cells grown in 5 $\mu\text{g}/\text{ml}$ of 5-bromodeoxyuridine alone for 3 days had about 3-5 times greater activity than untreated controls. Addition of thymidine to such cultures reduced the rise in activity, in parallel with graded concentrations of the natural nucleotide. When using double or greater amounts of thymidine (10-60 $\mu\text{g}/\text{ml}$) in combination with the analog, there was observed a nearly perfect block of alkaline phosphatase induction. These findings clearly indicate that thymidine does suppress the 5-bromodeoxyuridine effect.

In place of thymidine, several pyrimidine bases or pyrimidine nucleosides, such as cytosine, uracil, thymine, cytidine, uridine, deoxycytidine and deoxyuridine at a 4-fold higher concentration than that of 5-bromodeoxyuridine, were combined with the analog. None of them, however, was able to prevent the induction.

TABLE I

SUPPRESSION OF ALKALINE PHOSPHATASE INDUCTION BY SIMULTANEOUS ADDITION OF THYMIDINE WITH 5-BROMODEOXYURIDINE

Cells were cultured for 3 days in media containing either 5-bromodeoxyuridine alone, thymidine alone, or both, at the indicated concentrations.

5-Bromo- deoxyuridine ($\mu\text{g}/\text{ml}$)	Thymidine ($\mu\text{g}/\text{ml}$)	Alkaline phosphatase activity (nmoles/h per mg protein)	
		Expt 1	Expt 2
—	—	98	91
5	—	302	437
5	5	247	305
5	10	136	221
5	20	111	126
5	40	93	111
5	60	78	104
—	60	72	93

Induction of alkaline phosphatase by 5-iododeoxyuridine and 5-bromodeoxycytidine

As with 5-bromodeoxyuridine, 5-iododeoxyuridine and 5-bromodeoxycytidine inhibit the production of hyaluronic acid by B-6 cells¹. Conversely, treatment of the cells with these thymidine analogs was also found to increase the levels of alkaline phosphatase. As seen in Table II, the activity was greatly elevated with increasing amounts of either drug added at up to a concentration of 5 $\mu\text{g/ml}$, and at concentrations of 10–25 $\mu\text{g/ml}$ the induction seemed to level off. In the case of 5-iododeoxyuridine, there was a 2-fold increase also in acid phosphatase activity in the cultures treated with more than 5 $\mu\text{g/ml}$. The influence of both drugs on the cell growth, as determined by the protein content, was not of too great an extent in agreement with the data obtained using 5-bromodeoxyuridine¹.

TABLE II

INDUCTION OF ALKALINE PHOSPHATASE BY 5-IODODEOXYURIDINE OR 5-BROMODEOXYCYTIDINE IN B-6 CELLS

Cells were treated for 3 days with either 5-iododeoxyuridine or 5-bromodeoxycytidine at the varying concentrations indicated, and the activity of both alkaline and acid phosphatase was assayed. In addition, cell growth was determined by the protein content in these cultures.

Drug added	Final concn ($\mu\text{g/ml}$)	Phosphatase activity (nmoles/h per mg protein)		Cell growth ($\mu\text{g/ml}$ of culture)
		Alkaline	Acid	
5-Iododeoxyuridine	—	46	1190	85.6
	1	306	1370	77.0
	5	578	2220	67.0
	10	484	1880	67.6
	25	406	2650	48.8
5-Bromodeoxycytidine	—	88	1150	86.5
	1	230	1330	82.5
	5	784	1470	66.0
	10	816	1600	72.3
	25	576	1560	78.0

Comparing the present results with the previous ones on the induction-dose relationship of 5-bromodeoxyuridine¹, both 5-iododeoxyuridine and 5-bromodeoxycytidine were demonstrated to induce the activity of alkaline phosphatase in B-6 cells, not only at the same range of drug concentrations but also to the same degree as the brominated pyridine. In contrast, neither 5-bromouracil nor 5-bromouridine was active at concentrations from 1–50 $\mu\text{g/ml}$.

DISCUSSION

5-Bromodeoxyuridine has many novel effects on cultured cells, for example; (1) radiosensitization following incorporation into cellular DNA^{16,17}, (2) suppression of several differentiated functions^{4–10} or tumorigenicity¹¹, (3) induction of neuroblastoma differentiation¹⁸, *etc.* Also, in our previous and present studies, the thymidine analog was demonstrated to induce the alkaline phosphatase activity in the B-6 line.

The phosphatase has been described to be induced by the cultivation of some

cell lines in the presence of prednisolone¹⁹, substrates²⁰, or under unusual growth conditions like hyperosmolarity²¹ and low temperature²². However, not all cultured lines respond to such factors. In fact, the alkaline phosphatase in the cells used here is not affected by these factors, suggesting that the mechanism of the induction by 5-bromodeoxyuridine may differ from that of the above-mentioned factors.

The B-6 line was obtained by fusion of mouse mammary carcinoma cells and Chinese hamster lung cells *in vitro*². In examining the inducibility of alkaline phosphatase by 5-bromodeoxyuridine, only the parental mouse cells were found to respond to the analog. Therefore, it seems likely that B-6 cells have inherited the property from the mouse line.

There is much evidence for the toxic effect of 5-bromodeoxyuridine on the growth and survival of cultured cells²³⁻²⁶. In the presence of 5 $\mu\text{g/ml}$ or less, however, the B-6 line was able to multiply at a nearly normal growth rate during a prolonged period of incubation (18 days). When we surveyed a variety of cell lines in culture with respect to the inducible property of the phosphatase, the toxicity of the analog to cell multiplication was observed to differ greatly in each of the cell types: in medium containing 5 μg 5-bromodeoxyuridine per ml for 3 days of incubation, some lines propagated normally but others showed a decrease in growth rate and began to degenerate. From these comparisons, the B-6 line is judged to be relatively resistant to 5-bromodeoxyuridine.

The kinetics of induction were characterized by a considerably long lag time (24 h) prior to elevation in activity after adding 5-bromodeoxyuridine, and by a longer period (6-9 days) to reach a maximal level of induced activity in the presence of the analog. In HeLa cells, the rise in alkaline phosphatase levels by treatment with cortisol is likely to appear 12-24 h after exposure to the hormone and to cease at a new plateau within 60-100 h²⁷. Furthermore, in the case of the induction of the same enzyme with phenylphosphate in human fibroblast cells²⁰, a lag period of as long as 5 days occurs before the increase in activity begins, and a much longer period of more than 10 days to produce a maximum activity. On the other hand, the induction of tyrosine aminotransferase in rat hepatoma cell cultures²⁸ or glutamine synthetase in chick retina cultures²⁹ by dexamethasone or hydrocortisone has been shown to generally occur even about 2 h after treatment, reaching a new plateau in 5-8 h in the former case. Therefore, the response of alkaline phosphatase to inducers may generally be slower in time than that of other enzymes. At the present time when little is known about the mechanism of induction in our system, however, the reason remains unsolved.

The alkaline phosphatase induction was completely reversed by transfer and subculture of 5-bromodeoxyuridine-treated cells in the medium without the analog. 5-Bromodeoxyuridine is well known to be a mutagen in microbial cells, but in mammalian cells there is no evidence for such mutagenicity. The experimental results show that the very large increase in enzyme activity occurs after a short lag period (24 h), as compared to the generation time (16 h) of B-6 cells, and that the reaction is reversible, thus the induction should not be attributed to the mutagenic effect of 5-bromodeoxyuridine.

During the course of reduction of elevated alkaline phosphatase activity in the cultures treated for 3 days, there was a further increase in activity during a further 2 days after removal of the analog. Generally, enzyme induction by hormones

or substrates stops immediately at the time of removal of those inducers and begins to disappear rapidly by the decay of induced enzyme proteins due to their turnover rates, and their dilution due to cell multiplication. Nevertheless, the fact that further induction continues for 2 days even after transfer of treated cells to the analog-free medium, suggests that the inducer (5-bromodeoxyuridine) is tightly fixed to the cells in some way so as not to be diluted out easily. It seems, therefore, likely that the analog acts on the cells by virtue of incorporation into DNA, as we will discuss later. In contrast, the fully-induced activity after 15 days of successive treatment with 5-bromodeoxyuridine began to drop from the next day after removal of the analog. To explain this behavior, more studies need to be performed.

The B-6 line has the ability to produce hyaluronic acid³, and the synthesis is reversibly suppressed by 5-bromodeoxyuridine⁴. Thus, treatment of the cells with the analog causes the simultaneous induction of alkaline phosphatase and inhibition of the mucopolysaccharide, and this gave us an opportunity to examine precisely a relationship between these two, rather inverse effects of 5-bromodeoxyuridine. Consequently, we recognized that a close correlation exists between them in the following points: (1) The two effects are generally found by treatment with 5-bromodeoxyuridine, 5-iododeoxyuridine and 5-bromodeoxycytidine. (2) The effective concentration of 5-bromodeoxyuridine to give both the maximal inhibition of hyaluronic acid synthesis and the maximal induction of phosphatase is 5 to 10 $\mu\text{g/ml}$. (3) The two responses are reversible, and the recovery curves toward their normal levels proceed gradually during 3 transfer generations (9 days) with a lag period of one generation (3 days). (4) The addition of thymidine at concentrations 2-4 times greater than that of 5-bromodeoxyuridine along with the analog suppresses completely the two effects. (5) None of the other pyrimidine bases or nucleosides examined can replace thymidine in suppressing both effects of 5-bromodeoxyuridine. (6) Both hyaluronic acid synthetase involved in the terminal step of its biosynthesis³⁰ and alkaline phosphatase³¹ are likely to be mostly located on the plasma membrane or the large membrane fractions of cells.

On the basis of these similarities, it is strongly suggested that the two reactions, irrespective of their inverse nature, are caused by the same or common mechanisms during growth of B-6 cells with 5-bromodeoxyuridine. It is therefore probable that the suppression of hyaluronic acid synthesis in the present system, or of other differentiated functions by the analog, may be understood more easily and precisely through the elucidation of the mechanism of alkaline phosphatase induction.

Concerning the mechanism of 5-bromodeoxyuridine effects on differentiated functions, some workers have reported the requirement of incorporation of the analog into DNA in place of thymidine^{32, 33}. However, Schubert and Jacob¹⁸ showed that the differentiation of mouse neuroblastoma cells *in vitro* is induced by 5-bromodeoxyuridine even in the absence of DNA synthesis. We recently tried to solve these questions by using the induction of alkaline phosphatase and obtained some data supporting the necessity of incorporation of the analog into the B-6 cell DNA before it exerts an effect. In addition, the long duration of the effect of 5-bromodeoxyuridine after its removal on the induction as mentioned above, seems to support this view. These studies on the mechanism of the 5-bromodeoxyuridine effect will be reported elsewhere.

More recently, we have discovered that the alkaline phosphatase of the B-6

line is also induced by adenosine 3':5'-cyclic monophosphate or its dibutyl derivative³⁴.

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INDUCTION OF ALKALINE PHOSPHATASE BY CYCLIC AMP OR
ITS DIBUTYRYL DERIVATIVE IN A HYBRID LINE
BETWEEN MOUSE AND CHINESE HAMSTER IN CULTURE

Hideki Koyama, Ryoichi Kato and Tetsuo Ono

In some cultured mammalian cells, alkaline phosphatases (ALP) have been reported to be induced by prednisolone (1), substrates such as phenylphosphate or β -glycerophosphate (2), hyperosmolarity (3), and low temperature (4). Recently, we reported a pronounced induction of ALP by 5-bromodeoxyuridine (BUdR) in the hybrid line, B-6, derived from hybridization of mouse and Chinese hamster cell lines in vitro (5). This line has the inheritable ability to produce hyaluronic acid (6, 7), and the synthesis is inhibited reversibly by BUdR treatment (8).

During the course of studies on the mechanisms of the induction of alkaline phosphatase and inhibition of the mucopolysaccharide synthesis by BUdR, a striking rise in ALP activity was observed in response to cyclic AMP (c-AMP) or its dibutyryl derivative (DB c-AMP) added. The present paper describes the new phenomenon and some

results of experiments carried out for elucidation of this mechanism.

MATERIALS AND METHODS

The hybrid line (B-6) was grown in suspension cultures in a modified Eagle's medium (Nissui Seiyaku, Tokyo), supplemented with 5% calf serum and 0.1% Bacto-Peptone (Difco, Detroit) as described previously (6-8).

The methods for treatment of B-6 cells with chemicals and preparation of cell-free extracts for enzyme assay were the same as those cited in the preceding paper (5), except for the use of 50-mm Toyoshima plastic dishes (Toyoshima Seisakusho, Tokyo) containing 5 ml of the above medium and test compound in place of glass dishes. Alkaline and acid phosphatase activity of cell-free sonicates was measured by the method of Lowry (9). Specific activity was expressed as nmoles of p-nitrophenol released in 1 hr per mg protein. Protein was determined by the method of Oyama and Eagle (10) using bovine serum albumin as a standard.

The medium containing c-AMP or DB c-AMP was prepared by adding each drug to the medium and then sterilizing it with a Millipore filter just before use. c-AMP was purchased from Daiichi Pure Chem., Tokyo, and DB c-AMP from Boehringer Mannheim Japan, Tokyo. Actinomycin S₃, which inhibits RNA synthesis similarly to actinomycin D, was kindly supplied by Daiichi Pure Chem. Cycloheximide was obtained from Kaken Kagaku, Tokyo.

RESULTS

Table 1 indicates the alkaline phosphatase activity in the cells treated for 3 days with varying concentrations of c-AMP or DB c-AMP in the presence and absence of theophylline. c-AMP added in 0.4 mg/ml induced the activity to some extent, and cultures treated with 1 mg/ml had 2.8 times higher activity than the control. DB c-AMP was much more effective than the natural nucleotide. A marked induc-

Additions	Final concentration (mg/ml)	Alkaline phosphatase (n moles/hr/mg protein)	Acid phosphatase
None	—	55.3	1220
c-AMP	0.4	98.7	1160
c-AMP	1.0	196	1160
Theophylline	0.18	137	1060
c-AMP + Theophylline	0.4 0.18	413	1280
c-AMP + Theophylline	1.0 0.18	645	1280
None	—	48.8	1340
DB c-AMP	0.05	72.8	1480
"	0.25	186	1570
"	0.50	680	1720
"	1.0	3650	1920
"	2.5	14400	3060

Table 1. Effect of c-AMP and DB c-AMP on alkaline and acid phosphatase activity.
 2.5×10^5 viable cells were incubated for 3 days in various media in the presence of c-AMP, DB c-AMP or theophylline at the concentrations indicated.

tion was observed in parallel with the concentration of the derivative added. At the maximal concentration (2.5 mg/ml) examined, the activity was as much as 300-fold of the level of controls.

A lesser rise in the activity was also observed when the cells were grown in the medium containing theophylline (0.18 mg/ml), and the simultaneous addition of theophylline and c-AMP gave a higher level of activity than the additive value of activities due to each reagent employed separately. In contrast, acid phosphatase showed no significant changes in activity after the cells were cultured

with these chemicals except that DB c-AMP at higher concentrations produced some increase in the phosphatase level.

Increase in ALP was not observed 24 hr after addition of DB c-AMP (0.5 mg/ml), and thereafter a marked elevation proceeded linearly without showing any plateau even after 72 hr. Subsequently, following transfer of the induced cells to the drug-free medium, the high ALP level returned to the original in 3 days.

c-AMP is known to activate phosphorylase b kinase kinase (11), protein kinase (12), etc. as an allosteric effector. It was therefore expected that the same mechanism might be involved in the induction of alkaline phosphatase by c-AMP or DB c-AMP. As seen in Table 2, however, this possibility was excluded since the addition of these

Cell-free extracts	Additions	Alkaline phosphatase (nmoles/hr/mg protein)
I	—	35.6
II	—	324
I + II	—	189 (180)
I	Distilled water, 0.1ml	33.6
"	c-AMP, 0.4 mg/ml	31.2
"	c-AMP, 1 mg/ml	36.0
"	DB c-AMP, 0.5 mg/ml	33.6
"	DB c-AMP, 1 mg/ml	28.8

Table 2. Exclusion of the possibility for c-AMP or DB c-AMP to activate directly alkaline phosphatase or the presence of activators in the treated cells. Cell-free extracts, I and II, were prepared from the cultures grown for 3 days in the absence and presence of DB c-AMP at 0.5 mg/ml. To check the activation of ALP by the nucleotides, they were dissolved in distilled water, 0.1 ml of the solutions was added to the reaction mixtures at the final concentrations indicated, and the assay was run in the usual manner. On the other hand, to test the possibility of presence of activators in the treated cells, two cell-free extracts were mixed together in the same volume and the assay was carried out. The value in parenthesis is the arithmetical mean of both activities.

compounds to the enzyme solution prepared from control cultures did not result in the rise of activity. Besides, the activity of mixtures of cell-free extracts was equal to the arithmetical mean of both activities, suggesting that the elevation in ALP levels is neither due to activators in the cultures treated with both nucleotides nor to inhibitors in control cultures (Table 2).

The effect of actinomycin S₃ and cycloheximide on induction is represented in Table 3. At the concentrations used here, actinomycin S₃ inhibited RNA synthesis of the B-6 cells by more than 95%, while cycloheximide inhibited protein synthesis by more than 93% after one generation (16 hr). There was no change in the normal basal level of ALP activity 34 hr (two generations) after the addition of either of the antibiotics. In contrast, a 6.9-fold increase in activity with DB c-AMP (1 mg/ml) was completely prevented by actinomycin S₃. In the presence of cycloheximide, on the other hand,

Additions	Alkaline phosphatase	Acid phosphatase
	(nmoles/hr/mg protein)	
None	43.0	1240
Actinomycin S ₃	41.5	1170
DB c-AMP	689	1260
Actinomycin S ₃ + DB c-AMP	57.5	1150
None	38.0	1340
Cycloheximide	41.5	1150
DB c-AMP	698	1400
Cycloheximide + DB c-AMP	113	1140

Table 3. Effect of actinomycin S₃ (0.25 µg/ml) and cycloheximide (1 µg/ml) on the induction of alkaline phosphatase by DB c-AMP (1 mg/ml). 10⁶ viable cells were plated on dishes containing 5 ml of the medium with DB c-AMP and, at the same time, actinomycin S₃ or cycloheximide was added to them, followed by cultivation for 34 hr (two cell generations).

the induced activity was reduced to one-sixth, though this activity was still 3 times higher than the basal level. These data indicate that a new synthesis of both RNA and protein is required for c-AMP or DB c-AMP to induce alkaline phosphatase activity.

DISCUSSION

The present data show the induction of alkaline phosphatase activity by c-AMP and DB c-AMP in the B-6 line. Similar changes in the enzyme have also been found to occur in the same cells grown in BUdR (5). By comparing a variety of cells in culture with respect to the inducibility by DB c-AMP and BUdR, we have found that not all of them respond. These results will be reported elsewhere. On the other hand, with the B-6 cells, none of the factors described in the Introduction was effective, which had been reported to elevate ALP activity in some cultured cells (1-4).

Several experiments for elucidating the mechanism of induction by c-AMP and its derivative indicate that the response depends neither upon the activation of enzyme by an allosteric effect nor upon the formation of activators in the treated cells. Rather, there is some evidence for increased synthesis of enzyme protein, since actinomycin S_3 and cycloheximide block the induction completely or to a great degree.

Recently, Chader (13) reported that glutamine synthetase in chick retina cells in vitro is induced by c-AMP or DB c-AMP and that de novo enzyme synthesis is involved in the reaction because perfect blocking the induction was observed in the presence of both antibiotics used above. In contrast, DB c-AMP has been found to act at some posttranscriptional level to induce tyrosine aminotransferase in rat hepatoma cultures (14). Furthermore, Hsie and Puck (15) and Johnson et al. (16) observed drastic changes or transformation in morphology of some cultured cell lines, and the latter authors found no require-

ment of RNA synthesis for the response. c-AMP not only plays a well-defined role as a "second messenger" in the action of many hormones (17), but also has many effects in different biological systems, as well as in cultured mammalian cells. The mechanisms of these effects therefore will differ greatly in each system. More detailed studies in our system are now in progress.

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Fate of Mitochondrial DNA in Human- Mouse Somatic Cell Hybrids

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A considerable amount of evidence has accumulated in recent years indicating that, although mitochondrial DNA (mit-DNA) has a crucial role in the biogenesis of functionally active mitochondria, the nucleus provides a major part of the information required for the synthesis of mitochondrial constituents (see, for reviews, refs. 1 and 2). The existence of nuclear mutations in yeast that produce a "petite" phenotype (3, 4) is an example of evidence of such nuclear control. In animal cells, limitation of genetic tools has thus far hampered the analysis of the genetic control of mitochondrial structure and function. Recently, however, the development of the techniques of somatic cell genetics, and, in particular, of somatic cell hybridization, has opened the way to this type of analysis. Particularly promising in this respect appears to be the use of some interspecific somatic cell hybrids, which exhibit a preferential loss of chromosomes of one parental source. Thus, somatic cell hybrids between human and mouse cells, either from permanent cell lines or from freshly explanted diploid cultures, undergo a rapid and extensive loss of human chromosomes, while retaining all or almost all of the parental mouse chromosomes (5, 6). In the case of hybrids containing more than one complement of mouse chromosomes, the loss of human chromosomes is slower and less massive (7, 8). Recently, the isolation of an exceptional human-mouse hybrid clone exhibiting an apparently full complement of human chromosomes, but only a partial complement of mouse chromosomes, was reported (8).

In the present work, the fate of parental mit-DNA was investigated in several human-mouse hybrid cell lines. For this analysis, advantage was taken of the possibility of resolving mouse and human mit-DNAs on the basis of their difference in buoyant density in CsCl gradients. Only mouse-type mit-DNA was detected in all hybrid clones examined, even in hybrids estimated conservatively to contain an average of at least 23 residual human chromosomes per cell.

MATERIALS AND METHODS

Cells and Media. All cell lines were grown attached to glass or plastic tissue-culture bottles in the Dulbecco-Vogt modification of Eagle's medium [or in Ham's F12 medium (9) in the case of 3T3-4E] containing 10% calf serum, and further supplemented as specified below:

(a) Human cell line: Clone VA2-B, deficient in hypoxanthine:guanine phosphoribosyl transferase activity [derived from the line W1 18-VA2 transformed by Simian Virus 40 (10)]: it was grown in the presence of 3 $\mu\text{g/ml}$ of 8-azaguanine.

(b) Mouse cell lines: LM (TK⁻) Cl 1D, a subline of L cells deficient in thymidine kinase (11): it was grown in the presence or absence of 30 $\mu\text{g/ml}$ of bromodeoxyuridine (BrdU); another L cell derivative, A9, deficient in hypoxanthine:guanine phosphoribosyl transferase (12): it was grown in the presence of 3 $\mu\text{g/ml}$ of 8-azaguanine; 3T3-4 E, a permanent fibroblast line lacking thymidine kinase (7).

(c) Hybrid cell lines:

(i) Clones F and F6 from a cross between human diploid line FH10 and 3T3-4 E (8).

(ii) Clones E31 and F23 from a cross between VA2-B and Cl 1D (8): they were grown in medium supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT) (13) until 2-3 days before radioactive labeling, when aminopterin was eliminated. Clone F51 from the same cross, which grew very slowly in the presence of aminopterin, was maintained in medium containing hypoxanthine and thymidine alone. For all three clones, at the time of addition of labeled thymidine, the medium was changed to one supplemented with hypoxanthine only.

All hybrid cell lines used were isolated and analyzed by Jami *et al.* (8) in this laboratory.

Labeling Conditions. All isotopes were obtained from CEA, France. [Methyl-³H]thymidine (7 or 26 Ci/mmol; 0.33-0.83 $\mu\text{Ci/ml}$) was used to label DNA of A9 (for 96 hr) and

of the hybrid cell lines (for 48 or 72 hr). [Methyl- ^{14}C]-thymidine (55.3 Ci/mol; 0.020–0.070 $\mu\text{Ci/ml}$) was used to label the DNA of A9 and VA2-B cells (for 72 hr). DNA of Cl 1D cells was labeled with [5- ^3H]deoxycytidine (18 Ci/mmol; 0.33–0.41 $\mu\text{Ci/ml}$), and that of 3T3-4 E cells, with [2- ^3H]deoxyadenosine (10 Ci/mmol; 0.41 $\mu\text{Ci/ml}$), both for 72 hr.

Isolation of mit-DNA. Cells were detached from the bottles by trypsinization at 37°C for 5–10 min, washed twice with 0.01 M phosphate buffer (pH 7.0)–0.16 M NaCl (14), and swollen in hypotonic buffer for 45–60 min. Homogenization and differential centrifugation to isolate the mitochondrial fraction were described (15). The mitochondrial pellet was resuspended in 0.25 M sucrose–0.01 M Tris buffer (pH 6.7) (2.5 ml per 0.5–1.0 ml of the original volume of packed cells). Total mit-DNA was extracted from the pellet by a modification of the procedure of Smith, Jordan, and Vinograd (16). Briefly, after addition of MgCl_2 to 1.5 mM, NaCl to 0.01 M, and RNase and DNase, each to 100 $\mu\text{g/ml}$, the suspension was incubated at 2°C for 45 min, adjusted to 3 mM EDTA, and centrifuged at 10,000 rpm for 10 min in the Servall SS-34 rotor. The final mitochondrial pellet was resuspended in 2.4 ml of lysing buffer [0.01 M Tris buffer (pH 7.4)–0.01 M EDTA–1.2% sodium dodecyl sulfate]. When lysis was complete, Pronase was added to 75 $\mu\text{g/ml}$: after 30 min incubation at 37°C, the lysate was adjusted to 1 M CsCl, left at 2°C for about 60 min, and centrifuged at 15,000 rpm for 15 min in the Servall SS-34 rotor. The supernatant was centrifuged in the Spinco 50 rotor at 35,000 rpm for 16–17 hr. The pelleted nucleic acids were dissolved in 1.0 ml of 0.01 M Tris buffer (pH 7.4)–0.01 M EDTA, and total mit-DNA was separated from degraded nuclear DNA and RNA by centrifugation of this solution through a 2-step gradient (1 ml CsCl, $\rho = 1.76$, below 3.2 ml CsCl, $\rho = 1.40$, in 0.01 M Tris buffer (pH 7.4)–0.01 M EDTA–100 $\mu\text{g/ml}$ of ethidium bromide) in the SW 50L Spinco rotor at 38,000 rpm for 5 hr. Fig. 1a shows a typical pattern obtained from VA2-B cells labeled with [^{14}C]thymidine: the lower band represents closed-circular mit-DNA, the intermediate, open-circular mit-DNA, and the upper, degraded nuclear DNA. The fractions corresponding to the lower band were pooled and rerun to equilibrium in a CsCl–ethidium bromide density gradient (17) in the SW 50L rotor at 33,000 rpm for 48 hr. Fractionation and analysis of radioactivity led to a pattern like that shown in Fig. 1b. The lower band represents closed-circular mit-DNA, and the upper band, mostly open-circular mit-DNA arising from nicking of closed molecules. Only the lower band was used for further analysis.

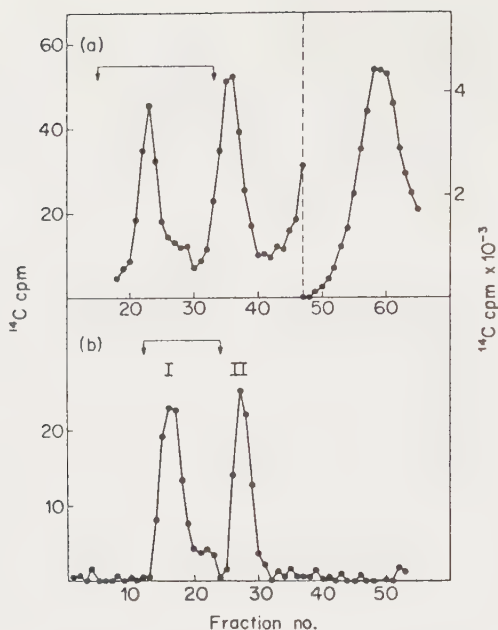


FIG. 1. Isolation of closed-circular mit-DNA from VA2-B cells labeled with [methyl- ^{14}C]thymidine. (a) Velocity sedimentation of total mit-DNA through a CsCl-ethidium bromide solution. (b) The fractions indicated by arrows in (a) were pooled and run to density equilibrium through a CsCl-ethidium bromide gradient.

The ethidium bromide was removed either by dialysis of the DNA samples against standard saline citrate [SSC: 0.15 M NaCl-0.015 M Na citrate (pH 7.0)] containing 1 mM EDTA for 3 days (18), or by passage through a Dowex 50W-X8 column (17).

Analysis of Density of mit-DNA in CsCl Gradients. Suitable aliquots of purified closed-circular mit-DNA in SSC containing 1 mM EDTA or in 0.01 M Tris buffer (pH 7.4)-0.01 M EDTA, from two differently labeled sources were mixed and brought to $\rho = 1.70$ with CsCl, usually in a total volume of 3 ml. Centrifugation was in the SW 50L Spinco rotor at 20°C for 72 hr at 33,000 rpm. 3-Drop fractions were collected from the bottom of the tubes directly on 25-mm paper filters, which were washed with trichloroacetic acid, ethanol, and ether, dried, and counted in a scintillation counter. (Samples in the peak regions were counted for 60 min or up to at least 500 counts). Several fractions throughout the gradient were collected in glass tubes for determina-

tion of their refractive index. The density (ρ) values reported in the figures have been determined by use of the equation:

$$\rho^{25^{\circ}\text{C}} = 10.860/n_D^{25^{\circ}\text{C}} - 13.497 \quad (19),$$

but have not been corrected for the small contribution to the density made by the buffer components.

RESULTS

Characteristics of hybrid clones

The origin and general behavior of the hybrid clones used in this work have been described (8). Table 1 shows the karyological characteristics of the parental and hybrid strains, based on examination of 20 metaphases for each, as they appeared within 1-2 days of the time when the cells were collected for the isolation of mit-DNA.

Clones F and F6, resulting from a cross of FH10 (carrying no selective marker) and 3T3-4E, and isolated in HAT selective medium, showed no evidence of the persistence of any human parental cells by karyological examination. Both these hybrids appeared to consist of cells with two sets (2S) of mouse chromosomes, and only a part of the original human chromosome complement (Fig. 2). On the basis of the excess of the total number of chromosomes over that expected for two sets of mouse chromosomes, one could estimate an average of 11 residual human chromosomes per cell in the case of the F line, and of 31 human chromosomes in the case of the F6 line. By use of the more stringent criterion of the number of metacentric chromosomes (thus not taking into account the possible residual human acrocentric chromosomes), the minimum estimate of human chromosomes would be an average of 8 for the F line and 17 for the F6 line.

The other three clones analyzed were obtained from a cross between VA2-B and Cl 1D, and were isolated in HAT selective medium. Two of these clones, which were maintained in HAT medium, contained full complements of mouse chromosomes (one set for F23 and two sets for E31) and were deficient in human chromosomes, having retained, on the average, six and 22-24 human chromosomes, respectively, as estimated on the basis of total chromosome number, or at least five and 19-23 human chromosomes, as estimated on the basis of the excess of metacentric chromosomes. The third clone, F51, at the start of growth for the preparation of mit-DNA, was unique in that it contained an apparently full complement of human chromosomes, while having only 6-30 mouse chromosomes. Since this clone grew very poorly in selective medium, aminopterin had to be removed. At the time of final karyological examination, clone F51 resembled

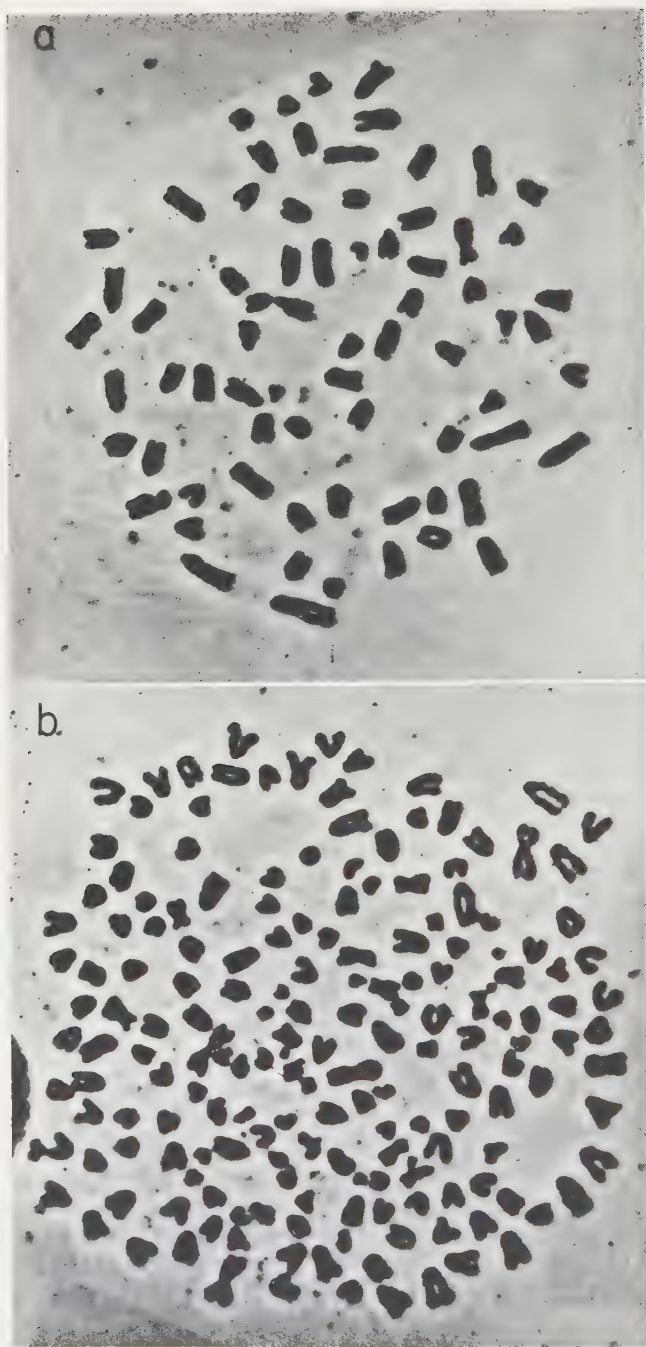


FIG. 2. (a) Mitotic figure of a 3T3-4 E parental cell containing 71 chromosomes. (b) Mitotic figure of an F6 hybrid cell containing in total 178 chromosomes, of which 16 are metacentric.

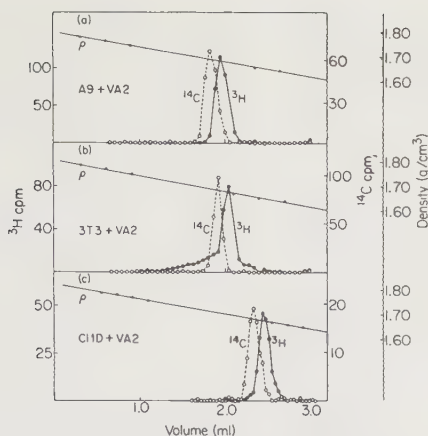


FIG. 3. Buoyant densities in CsCl of mouse and human mit-DNAs. Mixtures of [methyl- ^{14}C]thymidine-labeled VA2-B mit-DNA with [2- ^3H]deoxyadenosine-labeled 3T3-4 E mit-DNA (a), [methyl- ^3H]thymidine-labeled A9 mit-DNA (b), or [5- ^3H]deoxycytidine-labeled Cl 1D mit-DNA (c).

closely the parental VA2-B cells, but appeared to contain, on the average, about five more chromosomes per cell.

Resolution of human and mouse mit-DNAs by CsCl density equilibrium analysis

Previous reports (for summary, see ref. 20) had given an indication that human and mouse mit-DNAs differ enough in buoyant density so as to be separable in CsCl density gradients. CsCl density gradient centrifugation of a mixture of ^3H -labeled mit-DNA from mouse cells (3T3-4 E or A9) and ^{14}C -labeled mit-DNA of human source VA2-B, showed the mouse DNA to be reproducibly less dense by 0.008 g/cm^3 (Fig. 3a and b, respectively). Attempts to isolate closed-circular mit-DNA from Cl 1D cells (resistant to BrdU and grown routinely in the presence of $30 \mu\text{g/ml}$ of this drug to prevent reappearance of cells with thymidine kinase activity), with the use of widely different conditions of labeling, cell detachment and breakage, and of the isolation procedure for mit-DNA, were all unsuccessful. A small amount of apparently linear mit-DNA was obtained from Cl 1D under the conditions described in *Methods*, but this material gave a broad band in a CsCl gradient in the density region from $\rho = 1.70$ to $\rho = 1.73$, indicating that BrdU had been incorporated into mit-DNA. It was only upon the elimination of BrdU from the growth medium of Cl 1D that closed-circular mit-DNA could be isolated from this source; this DNA showed the same density difference with respect

TABLE 1. *Characteristics of the human-mouse hybrid and parental cell lines*

Cell line	Total number of chromosomes (a)	Total number of mouse chromosomes expected (b)	Estimate of number of human chromo-somes (a-b)	Total number of metacentric chromosomes (c)	Number of metacentric chromosomes expected from mouse parent (d)	Estimate of number of human metacentric chromo-somes (c-d)
<i>Parental</i>						
VA2-B	72.7 (67-80)	—	—	—	—	—
LM (TK ⁻) Cl 1 D	52.1 (47-54)	—	—	8.8 (7-11)	—	—
3T3-4 E	72.0 (67-82)	—	—	—	—	—
<i>Hybrid</i>						
(A) FH10 x 3T3-4 E						
F	155.1 (147-162)	144.0 (2 S)	11.1	8.4 (6-12)	—	8.4
F6	174.6 (157-195)	144.0 (2 S)	30.6	16.8 (10-25)	—	16.8
(B) VA2-B x Cl 1 D						
E31 _I *	128.3 (114-144)	104.2 (2 S)	24.1	36.5 (26-51)	17.6 (2S)	18.9
E31 _{II} *	126.1 (105-144)	104.2 (2 S)	21.9	40.6 (33-48)	17.6 (2S)	23.0
F23	58.2 (52-65)	52.1 (1 S)	6.1	13.5 (9-17)	8.8 (1S)	4.7

* Cultures independently grown from the same original stock for 24 (I) and 40 (II) days.

to the VA2-B mit-DNA marker (0.008 g/cm³) (Fig. 3c), as did the other mouse cell lines. A9 mit-DNA was chosen to serve as a marker in the analysis of mit-DNA of the human-mouse hybrid cells (see below), since it could be conveniently labeled with [methyl-¹⁴C]thymidine.

Analysis of mit-DNA from hybrid strains

Using ¹⁴C-labeled mit-DNA from A9 cells as marker, we examined the hybrid cell lines listed in Table 1 for the presence of mouse and human mit-DNAs. Fig. 4 shows the type of mit-DNA present in the two hybrid cell lines resulting from the FH10 x 3T3-4E cross. In both cases, the radioactivity of the hybrid mit-DNA coincides almost perfectly with that of the mouse marker, despite the fact that the hybrid cells contain a substantial number of residual human chromosomes (in particular F6, with an average of at least 17 human chromosomes per cell). A similar result was obtained with clones E31 and F23 derived from the VA2-B x Cl 1D cross (Fig. 5a and b). Reconstruction experiments showed that the presence in the hybrid cells of 10 percent human mit-DNA could have been detected. Two cultures grown independently from clone E31 and the culture grown from clone F23, had, on the average, at least 19, 23, and 5 human chromosomes per cell, respectively. Clone F51, which at the time of labeling showed a karyotype similar to the parental VA2-B, with,

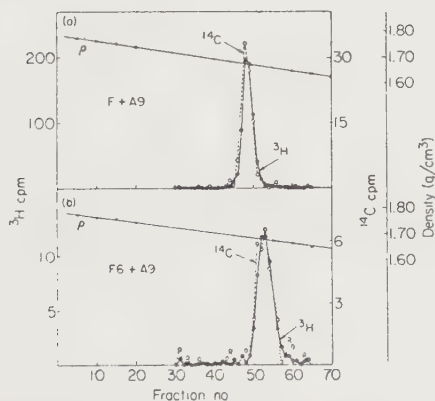


FIG. 4. Buoyant density in CsCl of mit-DNA from human-mouse hybrid cell lines, F and F6, derived from a cross between FH10 and 3T3-4 E. Mixtures of [methyl-¹⁴C]thymidine-labeled A9 mit-DNA with [methyl-³H]thymidine-labeled F (a) or F6 (b) mit-DNA.

however, an average of five chromosomes in excess over the parental number, contained only human-type mit-DNA (not shown); however, it is difficult to say, on the basis of the available data, whether the VA 2-B-similar cells were derived from hybrid cells having lost almost all the mouse chromosomes, or contaminating parental VA2-B cells, which took over the culture after the removal of aminopterin from the medium.

DISCUSSION

The main result reported here is that in human-mouse somatic cell hybrids still retaining a substantial number (up to an average of at least 23) of the original human chromosomes, only mouse-type mit-DNA was detectable. Two main explanations can be entertained for this result. The first is that the human nuclear and/or mitochondrial genes necessary for survival of human mit-DNA are repressed in the hybrid cell, independently of any chromosome loss, while the corresponding mouse genes are expressed normally. The second possibility is that the disappearance of human mit-DNA is a consequence of the loss of a part of the human chromosomes. Since cell hybrids at the initial stages after cell fusion, when little or no human chromosome loss has occurred, cannot be analyzed for technical reasons, it is not possible to distinguish between these two alternatives. This, however, should be feasible to test by the use of interspecific cell hybrids exhibiting little or no chromosome loss.

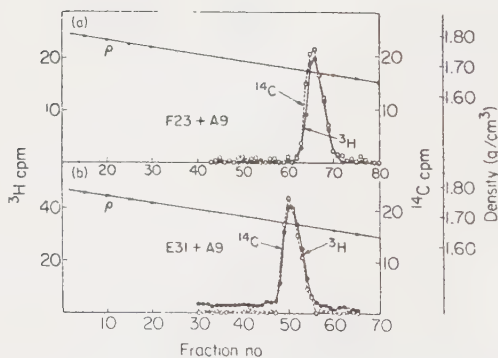


FIG. 5. Buoyant density in CsCl of mit-DNA from human-mouse hybrid cell lines, F23 and E31, derived from a cross between VA2-B and Cl 1D. Mixtures of [methyl- ^{14}C]thymidine-labeled A9 mit-DNA with [methyl- ^3H]thymidine-labeled F23 (a) or E31 (b) mit-DNA.

Since no obvious selective pressure existed for the retention of particular human chromosomes under the conditions used here [apart from the chromosome bearing the thymidine kinase gene (5, 21, 22)], it is likely that the loss of human chromosomes was substantially random. Therefore, if the disappearance of human mit-DNA is a consequence of the loss of part of the human chromosomes, to account for the present results one has to assume that the nuclear genes whose activity is essential for the survival of human mit-DNA are distributed in many chromosomes. This would not be a surprising result, since it is likely that replication of mit-DNA is coordinated with the growth and division of mitochondria, and since the greater part of the information for the synthesis of mitochondrial protein and lipid constituents is of nuclear origin. One would have to conclude, however, that mouse nuclear gene products cannot substitute, or substitute only in part, for the corresponding human nuclear gene products, and that, if any hybrid mitochondria are formed, they are selected against in the mitochondrial population.

We thank Dr. Boris Ephrussi for the hospitality of his laboratory and for his valuable advice and continued interest in this work, Dr. Mary Weiss for helpful discussions, and Mme. Natalie Riccio for technical assistance. This work was supported by the aid of the Délégation Générale à la Recherche Scientifique, a research grant from the U.S. Public Health Service (GM-11726), a postdoctoral fellowship from the American Cancer Society (PF-657) (B.A.), and a Guggenheim Fellowship (G.A.).

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THE RIBOSOMAL RNA OF HAMSTER-MOUSE HYBRID CELLS

GEORGE L. ELICEIRI

It has been shown that, in human-mouse hybrid cells, only mouse 28S ribosomal RNA (rRNA) can be detected, even in hybrids having up to 35 human chromosomes, including those believed to carry the rRNA genes (1, 2).

We have recently reported that in a hamster-mouse hybrid, T6aE-8A, both hamster and mouse rRNA were synthesized (3). The determination of hamster and mouse rRNA was based on two previous findings. First, Reader and Stanners (4) found that in the presence of 0.02 M Mg^{++} and 0.1 M K^{+} the majority of the free ribosomes of hamster sediment in a sucrose gradient as dimers, while those of mouse sediment mostly as monomers. Second, we found that hamster 28S rRNA migrates faster than mouse rRNA on polyacrylamide gel electrophoresis (5).

In the present report, the rRNA of a series of hamster-mouse hybrids (T6aE) has been studied by combining these two procedures. The ratio of hamster to mouse chromosomes varied within a 50-fold range among the 11 hybrid clones studied,

and the chromosome content, for each parental type, varied from about one-half haploid to about six times the haploid number of that particular species.

MATERIALS AND METHODS

The mouse parental cell line, 3T3-4E (6), the hamster parental cell line, T6a (7), and the hamster-mouse hybrids, T6aE (8), have been previously described. All cells were grown in Petri dishes in Dulbecco's modification of Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% bovine serum.

For determinations of hamster-mouse rRNA ratios, three semiconfluent 84 mm diameter plates of each cell type were incubated with uridine-5- 3H (about 25 Ci/mmol, 20–30 $\mu Ci/ml$ of medium) for about 24 hr, or with carrier-free ^{32}P (about 15 $\mu Ci/ml$ of medium) for 14–16 hr. The cells were harvested and the ribosome content was analyzed by sucrose gradient centrifugation, according to Reader and Stanners (4). Briefly, the cells were removed from the plates by incubation with 0.1% trypsin for 5 min at 37°C and washed with phosphate-buffered

saline (0.14 M NaCl, 0.003 M KCl, 0.02 M $MgCl_2$, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4); the pellet was resuspended in H buffer (0.1 M KCl, 0.02 M $MgCl_2$, 0.01 M Tris HCl, pH 7.4) and incubated in ice for 20 min. Brij-58 (Atlas Chemical Industries, Inc., Wilmington, Del.) was then added to a final concentration of 0.5%, and the cells were broken with a Dounce glass homogenizer. After spinning down nuclei and heavy debris for 5 min at 500 g, the supernatant was layered on top of a linear gradient of 15–30% sucrose in H buffer. The gradient was then centrifuged for 5 hr at 24,500 rpm and 4°C, in a Spinco SW25.1 rotor (Spinco Div. of Beckman Instruments, Inc., Fullerton, Calif.). Fractions of 0.65 ml were collected and samples were precipitated with 5% trichloroacetic acid, filtered, and counted by liquid scintillation. The ribosomal fractions from sucrose gradients were precipitated by addition of 0.1 vol of 20% potassium acetate, pH 5.1, and 1 vol of isopropanol. The resulting ribosomal pellets were then resuspended in 0.5% sodium dodecyl sulfate (SDS) and analyzed by 35 cm long, 2.4% polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis was carried out as described by Loening (9), with some modifications. Plexiglas tubing (Rohm and Haas Co., Philadelphia, Pa.) of 6–7 mm internal diameter was used, and the gels were 35 cm long. The composition of the gels was 2.4% acrylamide, 0.12% N,N' -methylenebisacrylamide, 0.04 M Tris-HCl, pH 7.2, 0.02 M sodium acetate, 0.001 M ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.2% SDS, 0.08% N,N',N'' -tetramethylethylenediamine, and 0.08% ammonium persulfate. The electrophoresis buffer, made up of Tris-HCl, pH 7.2, sodium acetate, EDTA, and SDS, in the same concentrations as in the gel, was recirculated throughout the run between the cathodic and anodic chambers with a pump. Electrophoresis was carried out at room temperature, at 5 ma/gel, for 50 hr. The desired portion of the gel was frozen with dry ice and fractionated with a Mickle gel slicer (Mickle Laboratory Engineering Co., England) into 1-mm thick slices. Each slice was incubated overnight at room temperature with 0.35 ml of toluene and 0.05 ml of NCS (Amersham-Searle Corp., Arlington Heights, Ill.). Each fraction was counted in a liquid scintillation counter after addition of 10 ml of scintillation mixture containing a 3/2 ratio of toluene and ethylene glycol monomethyl ether. For each electrophoresis the unknown sample was mixed with a hamster or mouse 28S rRNA marker labeled either with 3H or with ^{14}C , depending upon whether the unknown was labeled with ^{32}P or 3H , respectively.

Metaphase chromosome preparations were made by the method of Rothfels and Siminovich (10). From the modal numbers of acrocentric, telocentric,

and bi-armed chromosomes in each hybrid, the hamster and mouse complements were estimated as described by Basilico et al. (8). The extreme chromosome numbers were usually within $\pm 15\%$ of the modal numbers.

To determine the concentration of rRNA per cell, six semiconfluent 84 mm diameter plates of each cell type were grown for about 24 hr in the presence of uridine-5- 3H (about 15 $\mu Ci/ml$ medium, about 25 mCi/ $\mu mole$). The cells were then harvested in phosphate-buffered saline, counted in duplicate, and frozen in identical fractions. Some samples were then thawed, phenol extracted at 55°C (this was also done at room temperature), and their 3H cpm/OD 260 ratio was determined, before and after digestion with RNase-free DNase I (electrophoretically purified, Worthington Biochemical Corp., Freehold, N. J.), by polyacrylamide gel electrophoresis. The measurement of OD 260 in polyacrylamide gels was calibrated by quantitative addition of known samples of RNA to gels. Other samples were thawed and phenol extracted after being mixed with a series of identical aliquots of uridine- ^{14}C -labeled T6a whole cells, which had been grown and frozen in a similar way, independently. By measuring the ratio of $^3H/^{14}C$ trichloroacetic acid-precipitable counts, corrections could be made for losses during the phenol extraction of the 3H -labeled cells. Knowing the cell concentration, the specific activity of the RNA- 3H , and correcting for losses during handling by the $^3H/^{14}C$ ratio in RNA, it was possible to determine the relative concentrations of total RNA per cell among various cell clones. These values are relative to the phenol extraction conditions used in these experiments. By polyacrylamide gel electrophoresis of these fractions, it was found that 4S RNA represented about 16% of the total cell RNA for these various cells. As these samples had been grown in the presence of the isotope for about 24 hr, it was assumed that most of the remaining 84%, heavier RNA, was ribosomal. The reproducibility of the determinations of RNA concentration per cell was within $\pm 10\%$.

RESULTS

Fig. 1 shows sucrose gradient centrifugations of ribosomes of T6a and 3T3-4E. The cells were grown and harvested, and their ribosomes were analyzed as described in Materials and Methods. With the cell types and conditions used in these experiments, no significant amounts of polysomes were found, at least by the time the sucrose gradients were collected. Therefore, in the rest of this report attention is focused on ribosomes not bound to polysomes, as they represented essentially all the ribosomes found in these cells. Under these high concentrations of Mg^{++} and K^+ , most of

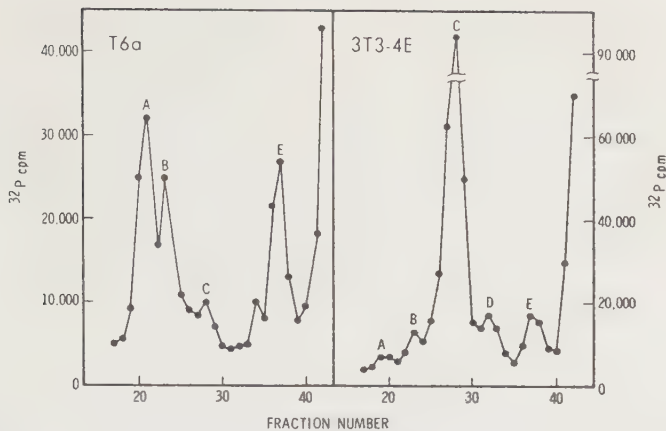


FIGURE 1 Sedimentation analysis of ribosomes from cultures of hamster parental cells (T6a) and mouse parental cells (3T3-4E). The conditions for isotope incorporation, preparation of cytoplasmic extracts, and sucrose gradient centrifugation were described in Materials and Methods. A, dimer; B, intermediate structure; C, monoribosome; D, 60S subribosomal particle; E, 40S subribosomal particle.

the ribosomes of the hamster parental cell line were present as dimers, a lesser amount as an intermediate, light structure, and a very small percentage as monomers. The amount of small ribosomal subunit (40S) was larger than that of 60S subunit. Fig. 1 also shows that most of the ribosomes of the mouse parent were present as monomers, with small amounts of the intermediate structure, dimers, and subunits. The intermediate structure of T6a in Fig. 1 seems to be made up of two 60S subunits and one 40S subunit, on the basis of the proportion of its 28S and 18S rRNA (G. L. Eliceiri, manuscript in preparation).

Figs. 2, 3, and 4 show the type of patterns obtained when the hybrid clones were analyzed by two successive steps, sucrose gradient centrifugation of ribosomes followed by polyacrylamide gel electrophoresis of rRNA, as described in Materials and Methods. In some hybrids, like 10A (Fig. 2) or 7A (Fig. 3), the majority of the ribosomes were present as monomers. The 28S rRNA of monomers consisted mostly of mouse rRNA, while hamster 28S rRNA could be found in dimer and intermediate peaks (Figs. 2 and 3). In other hybrids, like 3B, most of the ribosomes were present as dimers (Fig. 4). The dimer and intermediate peaks consisted mostly of hamster 28S rRNA, while mouse rRNA could be found in the monomer peak.

The ratio hamster/mouse 28S rRNA from dimer, intermediate, monomer, and 60S peaks was measured in 11 hybrids. These hybrid clones ranged between 16 and 92% of hamster chromosomes, with modal numbers between 12 and 131 for hamster chromosomes, and between 9 and 94 for mouse chromosomes.

During the course of these experiments it was noticed that some of the hybrids lost chromosomes, and that as they lost chromosomes of one species they also contained fewer ribosomes of that species. Therefore, in each experiment the chromosome composition of the hybrid was determined the day of cell harvest (see Materials and Methods).

Fig. 5 shows the relationship between the percentage of chromosomes of one species and the percentage of rRNA of the same species. The error in the determination of percentage of hamster and mouse rRNA was usually within $\pm 2\%$. It appears that when the majority of the chromosomes in a hybrid was of hamster type, a disproportionately higher percentage of hamster rRNA was present in that hybrid. Conversely, when hamster chromosomes were in the minority, a disproportionately lower percentage of hamster rRNA was present.

The total amount of rRNA per cell was determined in a third portion of cells, the same day of the preceding experiments, as described in Mate-

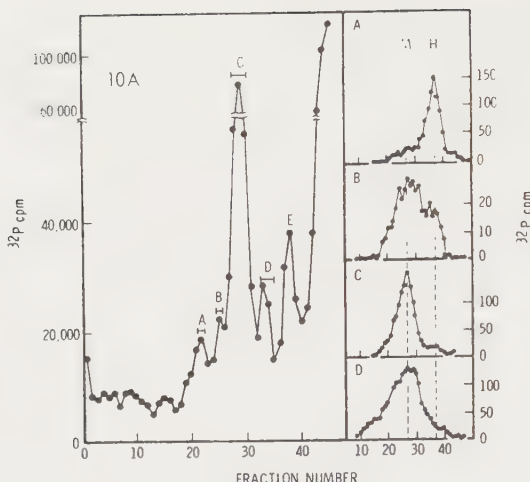


FIGURE 2 Sucrose gradient centrifugation of ribosomes from hybrid T6aE-10A (left panel) and electrophoretic analysis of 28S rRNA isolated from each sucrose gradient peak (right panels). The conditions of isotope incorporation, preparation of cytoplasmic extracts, sucrose gradient centrifugation, and polyacrylamide gel electrophoresis were described in Materials and Methods. *A*, dimer; *B*, intermediate structure; *C*, monoribosome; *D*, 60S subribosomal particle; *E*, 40S subribosomal particle. The locations of mouse (*M*) and hamster (*H*) 28S rRNA-³H markers, mixed with the unknown in the same electrophoresis gel, are indicated in the right panels (*A-D*).

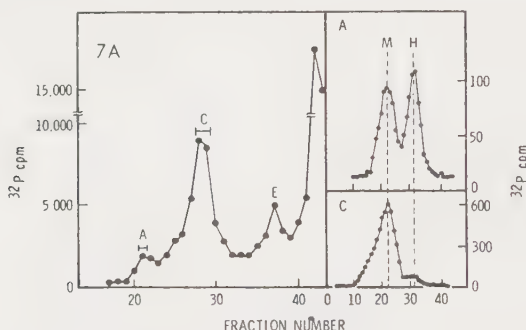


FIGURE 3 Sucrose gradient sedimentation of T6aE-7A (left panel) and electrophoretic analysis of its peaks (right panels, *A* and *C*). See legend of Fig. 2.

rials and Methods. The results are shown in Fig. 6, with linear extrapolations of the values found for the hamster parental cell (upper line) and for the mouse parental cell (lower line). The inset in Fig. 6 shows the same data expressed as micrograms of rRNA per milligram of total cell protein *versus* total number of chromosomes. Most hybrids have concentrations of rRNA per cell and rRNA

per total cell protein higher than could be expected by linear extrapolation of the rRNA concentration of 3T3-4E. The differences in content of rRNA per cell among various hybrids and T6a become negligible when the rRNA concentrations are expressed per total protein concentration.

Figs. 7 and 8 are obtained by combining the

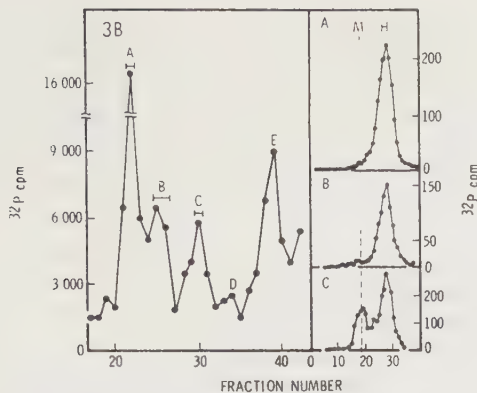


FIGURE 4 Sucrose gradient sedimentation of T6aE-3B (left panel) and electrophoretic analysis of its peaks (right panels, A-C). See legend of Fig. 2.

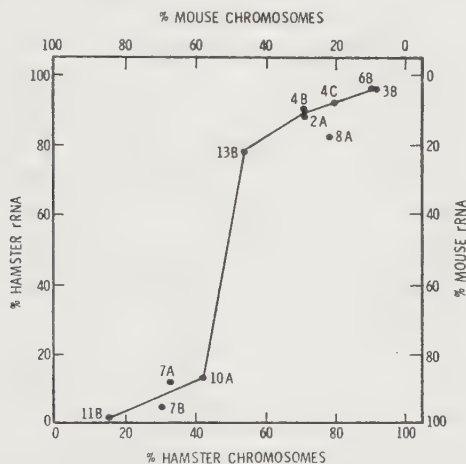


FIGURE 5 Comparison between the percentages of hamster and mouse rRNA per cell and the estimated percentages of hamster and mouse chromosomes in a series of T6aE hamster-mouse hybrids. The procedures to determine the ratio of hamster/mouse rRNA and the estimated modal numbers of hamster and mouse chromosomes were described in Materials and Methods and references 8 and 10.

data in Figs. 5 and 6. Fig. 7 shows the relationship between the hamster rRNA content and hamster number of chromosomes for each hybrid. Fig. 8 shows the equivalent comparison for the mouse species. In some hybrids the concentration of hamster rRNA per cell appears to be higher than the linear extrapolation of the T6a value (Fig. 7), but this difference disappears when the hamster rRNA concentrations are expressed per milligram

of total cell protein (inset of Fig. 7). There seems to be a sharp increase in the concentration of hamster rRNA per cell or per total protein when hybrids have over 70 hamster chromosomes.

In the case of mouse rRNA concentration, Fig. 8 shows that most hybrids parallel the linear extrapolation from the value of 3T3-4E. The exceptions are hybrids 7A, 7B, and 10A, in which the mouse rRNA concentration is approximately four

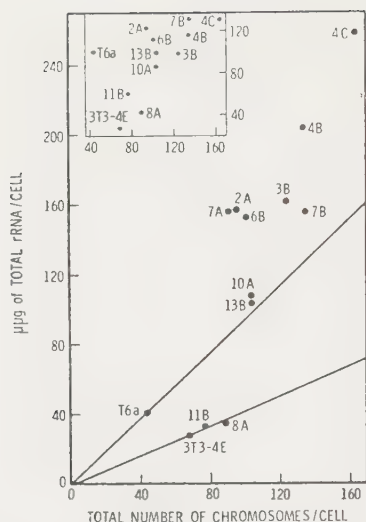


FIGURE 6 Comparison between the total concentration of rRNA per cell and the total modal number of chromosomes in a series of T6aE hybrids and their parental cells. The conditions to measure total rRNA concentration per cell and total number of chromosomes were described in Materials and Methods and reference 10. The inset shows the concentration of total rRNA per total cell protein (micrograms of rRNA per milligram protein in the ordinate) versus the total modal number of chromosomes (in the abscissa). The protein concentration of whole cells, broken with a Dounce glass homogenizer, was determined by the method of Lowry et al. (17), and the error limits were within $\pm 5\%$.

to five times higher than the concentration expected from that linear extrapolation. A relatively similar pattern is obtained when the data are expressed as mouse rRNA/total protein instead of mouse rRNA/cell (inset of Fig. 8). It is yet to be learned if these differences in concentrations of hamster or mouse rRNA are due to differences in the levels of RNA synthesis or degradation.

DISCUSSION

It has been shown that the cellular content of total RNA, rRNA, and ribosomes is two times higher in exponential growth phase than in stationary phase (11-14). As the present work was done with growing cultures, it would be interesting to study the rRNA content and hamster/mouse ratio of stationary cultures of these hybrids.

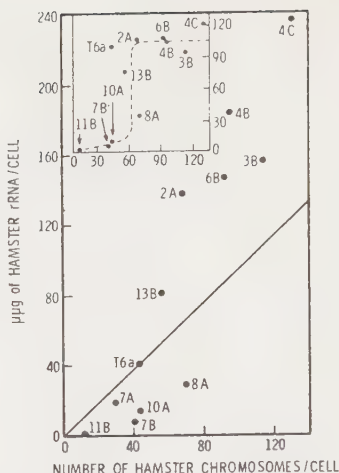


FIGURE 7 Comparison between the hamster rRNA concentration per cell and the estimated modal number of hamster chromosomes in a series of T6aE hybrids and the hamster parental cell line (T6a). This graph was made from data in Figs. 5 and 6. The linear extrapolation from the value for T6a is shown. In the inset, the ordinate is micrograms of hamster rRNA per milligram of total cell protein, and the abscissa is the estimated modal number of hamster chromosomes.

When the amount of rRNA of each species in hybrid clones is related to the number of chromosomes of that species, it is seen that the majority of the rRNA originates preponderantly from the genome contributing the majority of the chromosomes to the hybrid (Figs. 5, 7, 8). The result of this is that the minority genome does not contribute proportionately to the rRNA. This is shown for hamster chromosomes in Figs. 5 and 7, where there is an absolute depression of its contribution. In the case of mouse contribution there is also more than the proportional amount of mouse rRNA in clones possessing a large mouse genome (Figs. 5 and 8), and less than the proportional amount in clones possessing small complements (Fig. 5).

The mechanism of this effect can obviously not be explained until more is known about the means by which the synthesis of rRNA is regulated. One hypothesis would be as follows. The probability that any chromosome bearing ribosomal genes will form a nucleolus and transcribe rRNA would be about the same regardless of species. However,

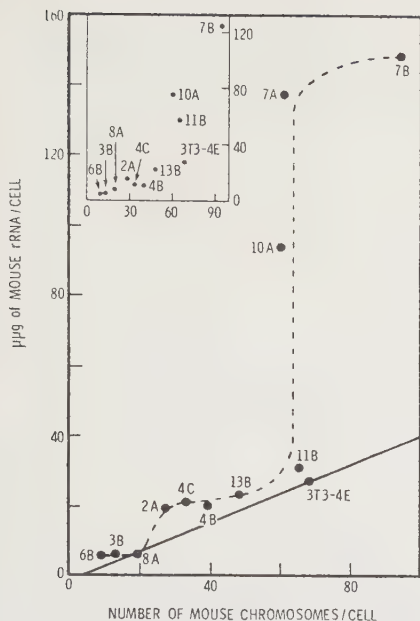


FIGURE 8 Comparison between the mouse rRNA concentration per cell and the estimated modal number of mouse chromosomes in a series of T6aE hybrids and the mouse parental cell line (3T3-4E). This graph was made from data in Figs. 5 and 6. The linear extrapolation from the value for 3T3-4E is shown. In the inset, the ordinate is microgram of mouse rRNA per milligram of total cell protein, and the abscissa is the estimated modal number of mouse chromosomes.

once a nucleolus is formed, other chromosomes of the same species can associate with it much more readily than chromosomes of the other species and transcribe their ribosomal genes. By this means the species contributing the majority of chromosomes will be more likely to initiate nucleolus formation (proportionate to chromosome ratio) and much more likely to synthesize rRNA (disproportionate to chromosome ratio). It is known that in certain plant hybrids the nucleolus-organizing capacity of chromosomes of one parental species is suppressed (Nawashin, cited by McClintock [15]). In gooseberry-blackcurrant hybrids, blackcurrant chromosomes cannot form nucleoli, but after loss of gooseberry chromosomes this function is regained (16).

The phenomenon described here may explain the observation of others that, in these hybrids, a

rather small difference in chromosome balance between the two species is associated with a large difference in the permissiveness of the hybrid to polyoma virus, which depends on the mouse genome. If the resulting viral DNA synthesis permitted by different hybrids is examined (8), it is clear from the present work that the hybrids which support viral DNA synthesis very well have over 80% mouse rRNA, while those which support it very poorly have less than 20% mouse rRNA. In both respects, therefore, the effects of imbalance of the two genomes are exaggerated at the phenotypic level. The question of whether the origin of the hybrid rRNA actually determines viral permissiveness by effects on translation of viral messenger RNA would be worth investigation.

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Regulation of Specific Functions of Glial Cells in Somatic Hybrids

I. CONTROL OF S100 PROTEIN¹

PHILIPPE BENDA AND RICHARD L. DAVIDSON

In a previous study on the regulation of specialized functions in mammalian cells, hybrids between Syrian hamster melanoma cells and mouse fibroblasts were isolated, and they were found to contain neither melanin nor dopa oxidase activity. These results suggested that the control of melanin synthesis involves a diffusible regulator substance, the result of whose action is the absence of dopa oxidase (Davidson et al., '66, '68; Davidson and Yamamoto, '68). This result led to a number of questions, including the following: (1) are other specialized functions in mammalian cells controlled in the same manner as melanin synthesis; and (2) are all the specialized functions characteristic of a given cell type controlled coordinately.

In an attempt to answer these questions, experiments similar to the above were undertaken with differentiated cells of another type, namely glial cells derived from a chemically induced rat brain tumor (Benda et al., '68; Benda, '68a; Benda et al., in press). Hybrids between these cells and fibroblasts were isolated and analyzed for the specific functions of the glial cells.

Glial cells cultured *in vitro* are characterized by a number of specialized functions, among them the synthesis of S100 (Benda et al., '68; Benda, '68a), a protein which is specific to neural tissues (Moore,

'65; Levine and Moore, '65; Moore et al., '68). This protein is found in normal brain (Moore, '65; Levine and Moore, '65; Moore et al., '68), in which it has been localized in glial cells (Hyden and McEwen, '66), and also in brain tumors of man (Benda, '68b). The structure of S100 has been conserved in a wide variety of vertebrates with only small variation (Levine and Moore, '65). The protein is soluble, highly acidic, and has a molecular weight of 24,000 (Moore, '65). Studies of purified beef S100 suggest that it is made up of several subunits (Calissano and Rusca, '69; Dannies and Levine, '69).

In the present paper the analysis of the glial cell $\times$ fibroblast hybrids for S100 is reported. In the second paper of this series (Davidson and Benda, '70), the analysis of the hybrids for other functions characteristic of the glial cells, such as the inducibility of the enzyme glycerol phosphate dehydrogenase (DeVellis and English, '69), is presented and the coordination of the various functions is considered.

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Cell lines. The original glial cell tumor was induced in rat brain by N-nitrosomethylurea and was subsequently cloned *in vitro* (Benda et al., '68). One clone, C6, was found to produce a large amount of S100 in cell culture, and this clone has now been maintained *in vitro* for over two years (several hundred generations) with continued production of high levels of S100. A subclone of line C6, designated RG6A, which produces as much S100 as C6, was used in the hybridization experiments to be described. The RG6A cells were hybridized with cells of two permanent lines of bromodeoxyuridine resistant mouse fibroblasts, LM(TK⁻)cl.1D (Kit et al., '63) (hereafter referred to as cl.1D) and 3T3-4(E) (Matsuya and Green, '69). Lines of 2S glial cells (RG6², cl.1 and cl.2) resulting from fusions between 1S RG6A cells were also isolated, and cells of one of these lines (RG6²-2) were hybridized with cl.1D.

Hybridization techniques. The production and isolation of the hybrids involved the virus induced fusion of cells in monolayers (described in detail in Davidson, '69) and the use of the "half system" modification (Davidson and Ephrussi, '65) of Littlefield's ('64) selective system. In a typical experiment, 1.5×10^6 cl.1D cells and 10^3 RG6A cells were mixed in a 3 cm plastic Petri dish containing growth medium (Ham's F10 medium supplemented with 10% of fetal calf serum). After 24 hours the cells were treated with 100 HAU of ultraviolet inactivated Sendai virus and then exposed to HAT selective medium (growth medium plus hypoxanthine, 1×10^{-4} M; aminopterin, 4×10^{-7} M; and thymidine, 1.6×10^{-3} M (Littlefield, '64)). Twenty-four hours later the cells were collected by trypsinization and distributed into forty 6 cm plastic dishes containing HAT. The dishes were refed with HAT every five days. In this medium the cl.1D cells degenerate, but both the RG6A cells and RG6A $\times$ cl.1D hybrids survive and multiply. Colonies of two types distinguishable by cell size and morphology were picked from the selective medium and were shown karyologically to be either glial cells or hybrids.

The RG6 $\times$ 3T3 and RG6² $\times$ cl.1D hybrids were isolated in a similar manner.

The RG6² cells were isolated following Sendai virus treatment of a monolayer of RG6A cells with virus and cloning in HAT. Colonies composed of cells larger than RG6A cells were subcultured and were identified karyologically as 2S glial cells.

Immunological methods. The cells to be tested for S100 were grown in 200 ml glass bottles, with an inoculum size of 5×10^5 . In most of the experiments with the RG6 $\times$ cl.1D hybrids, the cells were harvested seven days after inoculation, even though the specific activity of S100 in the glial cells at seven days is only 50% of the level found in cells harvested several days later. The choice of this point, however, was imposed by the fact that the hybrids in the majority of bottles detach from the glass after seven days. In order to avoid errors which could result from examining cells at only one point in the cycle, an experiment was performed in which a large number of bottles were inoculated with hybrids. From the few bottles in which the hybrids remained attached, the cells were harvested at 12 days after inoculation. In the experiments with the RG6² and RG6² $\times$ cl.1D hybrids, the cells were harvested ten days after inoculation.

The cells were collected, either by trypsinization or by scraping with a rubber policeman, rinsed with Tris-isotonic NaCl buffer, containing 0.5 mM mercaptoethanol, pH 7.4 and resuspended in the same buffer. After several cycles of freezing (in dry ice-ethanol) and thawing, the extracts were centrifuged at 30,000 or 105,000 $\times$ G. The supernatants were assayed for S100 activity, using the quantitative method of complement (C') fixation of Wasserman and Levine, as later modified by Levine ('67). Protein concentrations were determined by the method of Lowry et al. ('51).

The S100 specific activity of RG6A was determined by using purified beef S100 protein as a standard. The concentration of soluble glial cell protein required for maximum C' fixation, which is considered as the point of antigen-antibody equivalence, was compared with the concentration of the standard necessary for maximum C' fixation. The specific activity was expressed as μ g S100/mg of soluble protein. When two concentrations of protein

in successive two fold dilutions give maximum fixation, the point chosen to represent antigen-antibody equivalence is the one closer to the region of antibody excess, i.e., the point of lower protein concentration. Successive measurements of the same sample show that the maximum C' fixation always occurs at the same dilution.

When cell lines other than RG6A were tested for S100, the concentration of soluble protein required for maximum C' fixation (Q max.) was compared to the concentration of soluble protein of RG6A which gave maximum C' fixation (R max.). The serological activity of the line tested

is defined as $\frac{R. \max.}{Q. \max.} \times 100$. Thus, by definition, the serological activity of RG6A is equal to 100. If an extract produces maximum C' fixation at the same concentration of protein as RG6A, its serological activity is 100, and if 20 times more protein is necessary to obtain maximum C' fixation with a given extract than with RG6A, the serological activity of the extract is 5. The ratio of the protein concentrations giving maximum C' fixation, expressed as serological activity, can be assumed to be the same as the ratio of the S100 specific activities of the extract tested and RG6A only when the amounts of C' fixed at the maxima by the two extracts are the same.

The antiserum to S100 was prepared following the injection into rabbits of pure beef S100 coupled to methylated bovine albumin. Both purified beef S100 and the

antiserum were generously supplied to us by Dr. Lawrence Levine.

RESULTS

The design of the hybridization experiments permitted not only the isolation of the hybrids but also the determination of the frequency of their formation. In the RG6 $\times$ cl. 1D hybridization experiments, the virus treated cells were diluted so that each dish received the equivalent of 4×10^4 cl.1D cells and 25 glial cells. After three weeks in the selective medium, 20 out of 30 dishes contained colonies of hybrid cells. Therefore, according to the Poisson distribution there was an average of one hybrid cell colony per dish or stated otherwise one out of approximately every 25 glial cells fused with a cl.1D cell to give a viable hybrid. There was also an average of 19 glial cell colonies per dish. The glial cells thus have a cloning efficiency of almost 80%. Several colonies of glial cells and hybrids were picked (from different dishes to insure their independent origin) and analyzed karyologically and immunologically.

In the other hybridization experiments, one out of approximately every 25 RG6A cells produced a viable hybrid with 3T3 cells, 6% of the 1S RG6A cells fused to produce viable 2S glial cells, and one out of approximately every 60 RG6²-2 cells fused with cl.1D cells to give a viable hybrid.

The contributions of the glial cells and fibroblasts to the karyotypes of the hybrids was analyzed karyologically (table 1). The RG6A cells have a *modal* chromosome

TABLE 1
Karyological characteristics of parental and hybrid lines¹

Number of chromosomes	Telocentric	Large metacentric	Large submetacentric	Small metacentric	Small submetacentric	Total
Cell line						
RG6A	18	0	4	14	6	42 (41-42)
cl. 1D	44	9	0	0	0	53 (51-53)
3T3	75	0	0	0	0	75 (74-79)
RG6 $\times$ cl. 1D	60	9	4	14	6	92 (85-95)
RG6 $\times$ 3T3	91,94	0	3,4	13	5	116 (109-120)
RG6 ²	34	0	8	25	12	79 (76-82)
RG6 ² $\times$ cl. 1D	60	12	8	24	9	114 (105-122)

¹ The values represent the modes for all the clones together of a given line or hybrid. The numbers in parentheses following the total chromosome number indicate the range within which 90% of the cells are found. Because the number of chromosomes of each type for the RG6² $\times$ cl.1D hybrids are distributed throughout the range without forming a distinct mode, the values given for these hybrids and for the RG6² parent are means and not modes.

number which corresponds to that of diploid rat cells. In the hybrids, the large and small subtelocentric and the small metacentric chromosomes of the rat can be distinguished from the mouse chromosomes and therefore serve as markers. The RG6 $\times$ cl.1D and RG6 $\times$ 3T3 hybrids contain the number and distribution of chromosomes expected on the basis of the summation of the (modal) parental karyotypes. There is clearly very little chromosome loss, in particular for the rat marker chromosomes. The 2S glial cells (RG6⁺) contain approximately twice as many chromosomes of each type as the RG6A cells. The RG6⁺ $\times$ cl.1D hybrids have a total chromosome number which is 15% lower than expected. This is due almost entirely to the absence of 25% of the expected telocentric chromosomes. It cannot be said whether the loss of telocentric chromosomes is at random or if it is predominantly from one or the other parent. However, it may be noted that approximately 90% of the expected rat markers are present in these hybrids.

The 1S and 2S glial cells, cl.1D fibroblasts, and glial cell $\times$ cl.1D hybrids were tested immunologically for S100 activity using an antiserum prepared against purified beef S100. The specificity of this antiserum towards S100 from the rat glial cells was tested by immunodiffusion and immunoelectrophoresis. In immunodiffusion studies extracts of RG6A gave a precipitation band which formed a line of coincidence with the precipitation band of beef S100. Since no spur was formed, it can be concluded that the protein detected in the glial cells is qualitatively similar to the purified beef S100. Immunoelectrophoresis revealed a single precipitation arc, indicating that the antiserum reacts with determinants of only one protein in the glial cells.

The quantitative determinations of S100 were performed by C' fixation. The determination of the specific activity of S100 in RG6A is shown in figure 1. In this experiment the concentration of soluble glial cell protein required for maximum C' fixation (90%) was 25 μ g/ml, and the concentration of purified S100 which gave maximum C' fixation was 45 m μ g/ml. Thus 25 μ g of soluble protein from the

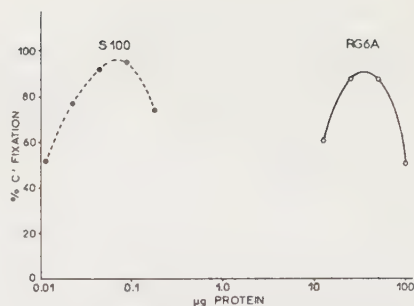


Fig. 1 Determination of S100 specific activity of RG6A: C' fixation curves obtained with purified beef S100 protein (●) and RG6A cell extracts (○), collected at seven days. Rabbit anti-serum 561, diluted 1:2,700.

RG6A cells contain 45 m μ g of S100, giving a specific activity of approximately 2 μ g of S100/mg of soluble protein. In other experiments, the specific activity varied between 1 and 2 μ g of S100/mg of protein.

The homogeneity of the RG6A population was examined by determining the S100 activity of seven subclones of RG6A which had been isolated from the same dishes as the RG6 $\times$ cl.1D hybrids. Since each clone was tested only one time, individual values will not be given. However, it was clear that all of the subclones had at least 50% as much S100 as the RG6A cells, and the average S100 specific activity of the seven clones taken together was almost identical to the specific activity of RG6A (an average of 94 compared to 100).

Tests of the C' fixing activity of cl.1D revealed a weak C' fixation (30%) at very high protein concentrations. There was no peak of C' fixation, but instead there was a plateau in the region from 500–1000 μ g of protein/ml (fig. 2). In addition to this weak fixation, anticomplementary effects were also observed; at concentrations of protein approaching 1 mg/ml, these extracts usually inactivated C' in the absence of antiserum in the antigen controls. When the antibody concentration was increased two to three fold, there was no shift in the concentration of protein which fixed C', but the amount of C' fixed was increased from 30% to 50%. This seems to indicate that the C' fixation is not an arti-

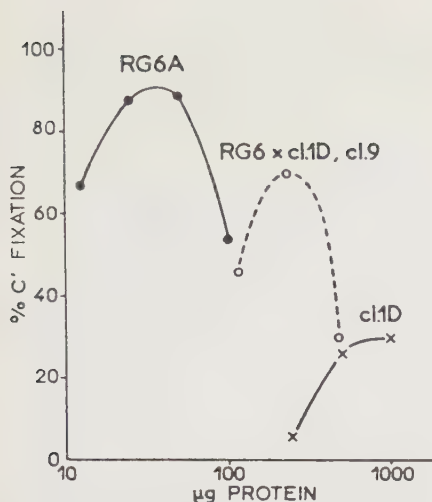


Fig. 2 Serological activity of parental cells and hybrids: C' fixation curves obtained with extracts of RG6A (●), cl.1D (×), and RG6 × cl.1D, cl.9 (○), collected at seven days. Rabbit anti-serum 561, diluted 1:2,700.

fact. The significance of this C' fixation by cl.1D will be considered below.

Seven different RG6 × cl.1D hybrids were tested for S100. At the time of their first analysis, these hybrids were approximately 30 generations old. The C' fixation curve for one hybrid clone (number 9) is shown in figure 2. The concentration of soluble protein which gave maximum C' fixation is nine times greater than that required for RG6A; thus the serological activity of the hybrid is 11. It can also be seen that the amount of C' fixed at the maxi-

mum by the hybrid was 20% less than that of RG6A. The significance of this will be discussed later.

The results of tests of all the RG6 × cl.1D hybrids, expressed as serological activity, are presented in table 2. The values for the hybrids range between 6 and 11. One of the hybrids (clone 9) was subcloned and four of its subclones were tested. All the subclones fixed C', and the average of the serological activities (which ranged from 8 to 19) was approximately the same as the activity of the parental clone (an average of 13, compared to 11 for clone 9). It should be pointed out that the amount of C' fixed at the maximum for all the hybrids is in the range of 50–70% in contrast to an amount of 75–90% for the glial cells.

It has been shown that the specific activity of S100 in glial cells increases in the post logarithmic phase of growth (Pfeiffer et al., '70; Benda, unpublished observations). Therefore hybrids were also examined late in the growth cycle. Two hybrid clones (RG6 × cl.1D, cl.1 and 9) were tested at seven days (as above) and at 12 days after inoculation in comparison to RG6A at the corresponding times. (During this period cells of the three lines undergo approximately one division.) For all three lines, approximately 50% less protein was necessary to obtain the point of maximum C' fixation with extracts of cells collected at 12 days than with extracts of cells collected at seven days. Thus there was a two fold increase in the serological activity of the hybrids as well as a doubling of the S100 specific activity of the glial cells between 7 and 12 days. The serologi-

TABLE 2
Serological activities in hybrid cell lines

Cell line	Activity ¹	Cell line	Activity	Cell line	Activity
RG6A	100	RG6 × cl.1D, cl.1	6	RG6 × cl.1D, cl.2	8
		cl.2	11	cl.3	10
RG6 × cl.1	135	cl.3	6	Average	9
cl.2	65	cl.4	6		
Average	100	cl.6	8		
		cl.9	11		
		cl.10	9		
		Average	8		

¹ The activity is given as the serological activity, as explained in Materials and Methods. All the values are expressed relative to the activity of RG6A, which is defined as 100. The RG6 × cl.1D hybrids were collected for analysis seven days after inoculation and compared with RG6A collected at the corresponding time. The RG6 × cl.1D hybrids were collected for analysis ten days after inoculation and compared with RG6A also collected at ten days.

cal activity of the hybrids expressed relative to the S100 specific activity of the glial cells at the corresponding time therefore remained the same.

Two clones of 2S glial cells were tested for S100 activity. One of the lines, RG6² cl.1, has a specific activity of S100 which is 35% higher than that of RG6A; and the other, RG6² cl.2, has an activity which is 35% lower (fig. 3, table 2). The amount of C' fixed by the two lines at the point of antigen-antibody equivalence is at least as much as that fixed by RG6A.

Hybrids between RG6² cl.2 and cl.1D were also tested. These hybrids fix complement at the same protein concentrations as RG6 × cl.1D hybrids, with serological activities of eight to ten (table 2). Moreover, the amount of C' fixed at the maximum is the same in the two types of hybrids.

DISCUSSION

One of the problems inherent in the use of somatic cell hybridization to study the regulation of specialized functions is the necessity of demonstrating that the results observed in the hybrids are not artifacts

of the hybridization system. A number of observations presented above suggest that the greatly reduced serological activity in the glial cell × fibroblast hybrids is not such an artifact: the S100 activity of the 2S glial cells RG6², which resulted from virus induced fusions between 1S RG6A cells and which were isolated by cloning in HAT, shows that neither exposure to virus and HAT nor cell fusion and the resulting increase in cell size and ploidy cause a decrease in S100 production; the S100 activity of all the RG6A subclones, together with the very high frequency of fusion of RG6A cells with fibroblasts, suggest that the hybrids did not result from fusions of fibroblasts with mutant glial cells which have low S100 activity; and finally the retention of essentially all of the rat marker chromosomes in the hybrids suggests that the reduced serological activity is not due to the loss of the relevant glial cell genes from the hybrids.

The results of the S100 assays for the glial cells, fibroblasts and hybrids are summarized in table 3, expressed in terms of both serological activity and activity/cell. The simplest interpretation of the results observed with the hybrids is that they contain S100 at an activity which is one tenth as high as that of the glial cells. If this were the case, the amount of C' fixed by the hybrids at the point of antigen-antibody equivalence should be the same as that fixed by the glial cells at the corresponding point, even though the protein concentrations necessary to reach this point are different. However, it has been shown that the hybrids fix on the average one third less C' at the point of maximum fixation than do the glial cells. This raises the possibility that the complement fixing material in the hybrids is not normal S100, but is instead either a modified S100 or a different, cross-reacting protein. It is clear that in the case of either a modified S100 or a cross-reacting protein, the significance of the measurements of serological activity become questionable. If the C' fixing material in the hybrids is a slightly modified S100, then the reduced serological activity in the hybrids would be explained by the presence of a small amount of this material, and the decreased amount of C' fixed would be explained by the slight

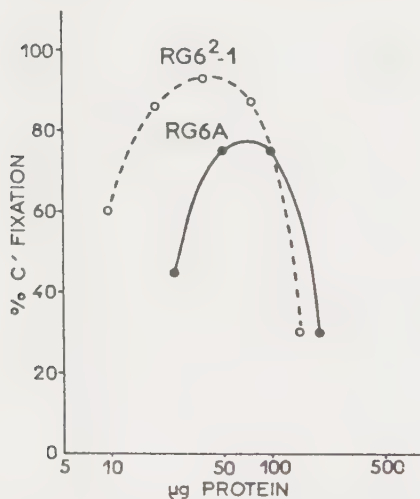


Fig. 3 S100 activity of 2S glial cells: C' fixation curves obtained with extracts of RG6 (●) and RG6²-1 (○), collected at ten days. Rabbit antiserum 246, diluted 1:1,000.

TABLE 3
Serological activities in parental and hybrid cell lines

Cell line ¹	Serological Activity	Protein/cell	Activity/cell ²
cl.1D	5	$1.3 \times 10^{-4} \mu\text{g}$	6
RG6 $\times$ cl.1D	8	2.4	17
RG6 ² $\times$ cl.1D	9	3.4	28
RG6 ² .cl.1	135	2.2	270
cl.2	65	2.2	130
RG6A	100	1.1	100

¹ This table gives the averages for all of the clones of a given class except for RG6². Since the two RG6² clones have very different values, the individual figures are given.

² Activity/cell is the serological activity multiplied by the protein/cell, and expressed relative to the value in the RG6A cells, which is set equal to 100.

modification in the protein. However, if the material in the hybrids is a highly modified S100, an increase in the antigen concentration necessary for maximum C' fixation and a decrease in the amount of C' fixed would be expected, even if the concentration of modified S100 in the hybrids were as high as that of S100 in the glial cells. A situation analogous to this has been described for the thermal denaturation of purified S100 at 50–60°C (Kessler et al., '68). It will be necessary to purify at least partially the C' fixing material from the hybrids in order to determine whether these cells contain a more or less modified S100 or a cross-reacting protein. It may be pointed out that this same remarks are relevant for the C' fixing material in the cl. 1D cells.

The decreased serological activity in the hybrids may be indicative of the operation of a mechanism used by the fibroblasts to control S100. If the C' fixing material in the hybrids is a cross-reacting protein and not S100, i.e., if the specialized function of the glial cells is not expressed at all after fusion with fibroblasts, then the results would agree with those found in studies of melanoma $\times$ fibroblast hybrids (Davidson et al., '66, '68; Davidson and Yamamoto, '68; Silagi, '67) and recently in studies of pituitary $\times$ fibroblast hybrids (Sonnenschein et al., in preparation). The results would then be consistent with the idea of the control of differentiated functions by diffusible regulator substances the result of whose action is the absence of the synthesis of specific proteins, as postulated in the studies on melanin synthesis (Davidson et al., '66, '68; Davidson and Yamamoto, '68). On the other hand, if there is

a small amount of normal or slightly modified S100, there could be a block in the hybrids which is only partial. (The possibility that a fraction of this S100 is synthesized by the genome of the fibroblast in the hybrids would have to be considered.) The absence or decrease of S100 in the hybrids could also be consistent with mechanisms involving alterations in surface properties, as suggested by the results of experiments in which glial cells were grown in mixed cultures with brain cells which did not synthesize S100 (Pfeiffer et al., '70). At the other extreme, if the hybrids contain a large amount of highly modified S100, the mechanism used to control S100 activity could involve a modification of the molecule after its synthesis. An assumption which is implicit in the last hypothesis is that a messenger RNA produced by the glial cell genome would be faithfully translated in the hybrids.

One of the factors complicating the interpretation of the results obtained with the hybrids is the presence of some C' fixing material in the cl.1D cells, since the relationship of this material to the activity detected in the hybrids is not known. It has recently been found that 3T3 cells do not contain any C' fixing material. The glial cell $\times$ 3T3 hybrids are presently being analyzed, and these tests may provide additional information on the regulation of S100.

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Interaction Properties of Cell Hybrids Formed by Fusion of Interacting and Non-interacting Mammalian Cells

By JILL McCARGOW and J. D. PITTS

Mutant BHK cells,* derived from the hamster fibroblast line BHK 21/13 (Macpherson & Stoker, 1962), that lack inosinate pyrophosphorylase or thymidine kinase (BHK-IPP⁻ and BHK-TK⁻ cells; Marin & Littlefield, 1968) are unable to incorporate [³H]hypoxanthine and [³H]thymidine respectively when grown separately in tissue culture. However, when grown together in confluent cultures both cell mutants incorporate [³H]hypoxanthine and [³H]thymidine. This mutual phenotypic modification of BHK-cell mutants, through cell-cell contact in tissue culture, is most probably explained by the transfer of [³H]purine nucleotides and [³H]thymidine nucleotides through intercellular junctions, which, unlike the normal cytoplasmic membrane, are permeable to these nucleotides (Bürk, Pitts & Subak-Sharpe, 1968; Pitts, 1971; J. D. Pitts, unpublished work).

Mutant L cells, derived from the mouse fibroblast line L929 (Sanford, Earle & Likely, 1948), namely L-IPP⁻ and L-TK⁻ cells (Littlefield, 1966), on the other hand do not interact in this way and remain phenotypically distinct when grown together in confluent cultures. Further, the inability of such mutant L cells to interact is dominant in mixed cultures of L cells and BHK cells (Pitts, 1971a).

The interaction properties of cell hybrids formed

between BHK cells, which do interact, and L cells, which do not interact, have now been studied. Hybrids were formed between BHK-TK⁻ cells and L-IPP⁻ cells by fusion with inactivated Sendai virus (Harris & Watkins, 1965) and selected by growth in medium supplemented with aminopterin, thymidine and hypoxanthine.

Cloned hybrid BHK-L cells were phenotypically wild-type, incorporating both [³H]hypoxanthine and [³H]thymidine, and interacted in mixed confluent cultures with mutant BHK cells but not with mutant L cells. The hybrid cells were shown to possess both BHK-cell and L-cell cytoplasmic membrane determinants by cytotoxicity tests with rabbit anti-(hamster cell) serum and rabbit anti-(mouse cell) serum, in the presence of guinea-pig complement, and measuring cell lysis by the uptake of Naphthalene Black.

These results are consistent with the concept that cells that fail to interact in tissue culture have lost some membrane function essential to both donor and recipient cells for the formation of the intercellular junctions required for nucleotide exchange.

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* Abbreviation: BHK cells, baby-hamster kidney cells.

Effect of 5-Bromodeoxyuridine on Hyaluronic Acid Synthesis of α Clonal Hybrid Line of Mouse and Chinese Hamster in Culture

HIDEKI KOYAMA AND TETSUO ONO

In previous papers, we reported the isolation and characterization of hybrids of mouse and Chinese hamster cell lines (Koyama, Yatabe and Ono, '70), and the expression of a highly differentiated function (HA synthesis) in one of these hybrid lines, designated B-6 (Koyama and Ono, '70). This function is considered to have been initiated by hybridization since both parental lines have little ability to synthesize the mucopolysaccharide. B-6 cells have continued to produce and secrete HA at a high, constant rate from the beginning of isolation for more than two years, and are easy to handle in either single or mass cultures since they grow in a floating state like ascites tumor cells. Thus, this system seems to be very useful for elucidating the control mechanism of expression of differentiated functions in mammalian cells.

Recently, the expression of differenti-

ated functions in chick embryo cells such as myogenesis in myoblasts (Stockdale et al., '64; Okazaki and Holtzer, '65; Coleman et al., '69; Bischoff and Holtzer, '70), chondroitin sulfate synthesis in chondrocytes (Abbott and Holtzer, '68; Coleman et al., '68; Lasher and Cahn, '69), HA synthesis in amnion cells (Bischoff and Holtzer, '68), melanin synthesis in retina cells (Coleman et al., '68), and in mouse melanoma cells (Silagi and Bruce, '70) has been shown to be inhibited by relatively low concentrations of BUDR, which does not interfere with cell multiplication. Also in the last paper, tumorigenicity of the melanoma cells has been shown to decrease by treatment with the analog. The purpose of this communication is to report on experiments undertaken to see whether BUDR has the same inhibitory effect on HA synthesis of an artificial, interspecific

hybrid line, and to examine its effect on cell growth. Furthermore, the effects of excess TDR and another analog, FUDR, on both functions will be compared with that of BUDR.

MATERIALS AND METHODS

Cell line and culture method

The clonal hybrid line, B-6, used here was derived from the mating of an 8-azaguanine-resistant FC-1 line of C3H mouse mammary carcinoma origin, and a CHL line established from newborn Chinese hamster tissue (Koyama, Utakoji and Ono, '70). Its properties were reported in previous papers (Koyama, Yatabe and Ono, '70; Koyama and Ono, '70). The cells were grown in 100 mm glass dishes in 10 ml of the standard medium consisting of modified Eagle's medium (Yamane et al., '68; Nissui Seiyaku, Tokyo), 5% calf serum, and 0.1% Bacto-Peptone (Difco, Detroit), in a 5% CO₂-incubator at 37°C. Subcultures were performed by transfer to fresh medium with appropriate dilution twice a week.

Treatment with BUDR or other agents

Cells harvested at the logarithmic phase of growth were collected by centrifugation at 1000 rpm for five minutes, washed once with fresh medium, and counted in a Thoma-type hemocytometer after staining in 0.25% trypan blue solution. The viable cells were inoculated at an inoculum size of 2.5×10^5 cells/dish into a series of 50 mm Toyoshima plastic dishes (Toyoshima Seisakusho, Tokyo) containing 5 ml of the standard medium with appropriate concentrations of BUDR or other agents. The cells were cultured in the CO₂-incubator covered with a dark paper for various periods depending on the aim of each experiment. Then, the cultures were transferred to small tubes and centrifuged at 1200 rpm for ten minutes. The medium of each culture was collected by decantation into new tubes and stocked at -20°C until the assay for the amount of HA was performed. At the time of decantation the viable cells and total number of cells in each culture were counted by means of trypan blue exclusion test as described

above. Each value in all the results was obtained by using three replicate dishes.

Estimation of the amount of HA secreted into the medium

The method was the same as that described in our previous paper (Koyama and Ono, '70).

Chemicals

BUDR and TDR were purchased from Sigma Chemical Co., St. Louis. FUDR and bovine testicular hyaluronidase were kindly supplied by Dr. A. Tsuya in our institute hospital and Dr. E. Mochida, Mochida Pharm. Co., Tokyo, respectively.

RESULTS

Effect of BUDR, TDR, and FUDR on cell growth and HA synthesis

First, the effect of each reagent on the growth and HA synthesis of B-6 cells was studied by treatment of the cells with varying concentrations of the drug for three days. The relative growth rates of treated cultures and the relative amount of HA produced by them were expressed as percentages of those in control cultures.

Figure 1a shows that the presence of BUDR at concentrations of 0.5 to 1 $\mu\text{g/ml}$ had no effect on the multiplication of the cells, but that at higher concentrations some inhibitory effect appeared. Treatment with concentrations ranging from 5 to 25 $\mu\text{g/ml}$ resulted in a constant decrease of approximately 15% in the number of total cells or 30% in that of viable cells.

In contrast, the effect of the analog on the production of HA differed significantly from its effect on the growth. The amount of HA produced by the cells treated with BUDR at 0.5 or 1 $\mu\text{g/ml}$ was reduced to about 75 or 60%, respectively, of the control level. This reduction was more pronounced at the higher concentrations. The cultures grown in 5 to 25 $\mu\text{g/ml}$ of BUDR were able to secrete only 27% HA on the average, as compared with the untreated cultures, so that the BUDR treatment diminished the amount of the mucopolysaccharide synthesized per cell to about one-third of the level of the control. These results clearly indicate that BUDR inhibits

HA synthesis either more strongly than or in preference to the multiplication of B-6 cells, in agreement with the data reported previously by Bischoff and Holtzer ('68) for chick embryo amnion cells.

TDR is a normal metabolite of cells, but is known to inhibit their DNA synthesis or growth at higher concentrations. When added to the medium at up to 24 $\mu\text{g}/\text{ml}$, TDR did not affect the growth or HA synthesis of B-6 cells, but over that concentration a gradual, inhibitory action on both functions was observed with increasing amounts of the metabolite (fig. 1b). In

this case, one apparent finding was that the inhibited rate of HA production by TDR was always smaller than the inhibition of the growth rate, in contrast to the case of BUDR. Similar but more definite data were obtained by the treatment of the cultures with another analog, FUDR (fig. 1c). Although cultivation in less than 0.001 $\mu\text{g}/\text{ml}$ of the analog was followed by normal synthesis of HA as well as normal cell proliferation, over that concentration their growth was suppressed more markedly than the production of the mucopolysaccharide. Though net increase in cell number did not occur in the presence of 1 $\mu\text{g}/\text{ml}$ of FUDR, more than 50% of the ability to accumulate HA in medium was retained.

Several other halogenated pyrimidines were tested within the same concentrations as BUDR (at 25 $\mu\text{g}/\text{ml}$ or less). Among them, 5-iododeoxyuridine and 5-bromodeoxycytidine showed the same inhibitory effect on HA synthesis as BUDR. Neither 5-bromouracil nor 5-bromouridine had any effect on either function.

Time course of the effect of BUDR, excess TDR, and FUDR

B-6 cells were cultured in the standard medium containing each test agent, and then their growth and production of HA were assayed every day as described in Materials and Methods. The results are shown in figure 2. The amount of the mucopolysaccharide secreted by the 10^6 cells treated with 5 $\mu\text{g}/\text{ml}$ of BUDR decreased gradually with incubation time. In the first day it was almost the same as in the control, but was reduced to approximately one-half and then one-third of the control level (24.8 $\mu\text{g HA}/10^6$ cells on the average) on the second and third days, respectively (fig. 2a).

On the other hand, cells grown in either excess TDR (240 $\mu\text{g}/\text{ml}$) or FUDR (0.1 $\mu\text{g}/\text{ml}$) for more than two days were found to produce HA at a more rapid rate than untreated cells (figs. 2b, 2c), though there was no significant effect on the first day. In the case of excess TDR, the increase in the rate of HA synthesis was enhanced with time, reaching as much as 2.5 times the control rate on day 4. Similarly, FUDR-treated cells were able to accumulate about

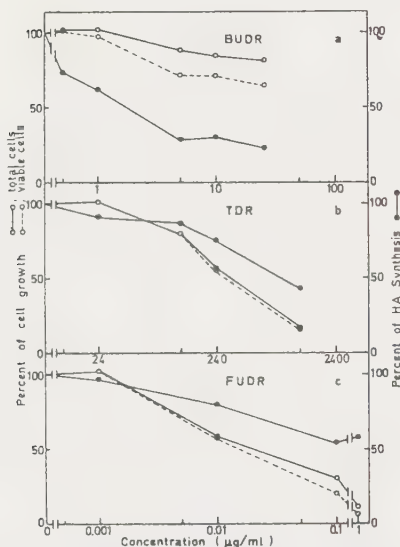


Fig. 1 Effect of BUDR, TDR, and FUDR on the growth and HA synthesis of B-6 cells as a function of concentration. 2.5×10^5 cells were grown for three days in the standard medium containing each agent in 50 mm plastic dishes. Cell number and the amount of HA in the medium were determined as described in Materials and Methods. The result is expressed as relative percentages of the control values. Untreated control cultures yielded an average of 3.4×10^6 total cells per dish after three days (7 experiments), their viability as checked by the trypan blue exclusion test was more than 95%, and the mean amount of HA produced per 10^6 total cells was 23.9 μg . All the points in figures 1a, 1b and 1c are the means of 3, 2, and 2 experiments, respectively.

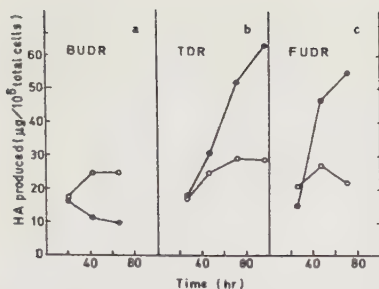


Fig. 2 Time course of the effect of BUDR, excess TDR and FUDR on the synthesis of HA. The experimental conditions were the same as those indicated in figure 1, except for assaying the number of total cells and the amount of HA every day. The result is expressed as the amount of HA produced per 10^6 total cells. $\circ$ — $\circ$, control cultures; $\bullet$ — $\bullet$, cultures treated with BUDR (5 $\mu\text{g/ml}$), TDR (240 $\mu\text{g/ml}$), or FUDR (0.1 $\mu\text{g/ml}$).

twice the amount of mucopolysaccharide per cell on the second to third days, as compared with the control.

These data confirm that BUDR inhibits HA synthesis more than cell growth while showing that both excess TDR and FUDR produce just the opposite effect on BUDR. All these influences are detectable after two days of incubation with each of the reagents under the present experimental conditions.

Prevention of the effect of BUDR by the addition of TDR

BUDR is known to be metabolized by thymidine kinase, the enzyme responsible for a first-step phosphorylation of TDR. Therefore, the addition of TDR along with BUDR was expected to counteract the

effect of the analog. The result of a typical experiment is represented in table 1. The total cell number and the amount of HA in the cultures grown in 5 $\mu\text{g/ml}$ of BUDR for three days were reduced by about 24% and 61%, respectively, and this reduction was hardly affected by the addition of the same concentration of TDR as the analog. It was, however, found that the addition of double the amount of TDR (10 $\mu\text{g/ml}$) completely prevented growth inhibition, while the BUDR-treated cells produced the mucopolysaccharide as much as 88% of the control. Furthermore, the presence of four-fold concentrations of TDR (20 $\mu\text{g/ml}$) with BUDR restored not only the normal cell proliferation, but also the synthesis of HA up to more than 94% of that in the control cultures.

Subsequently, nucleosides various pyrimidines or pyrimidine such as cytidine, uridine, deoxycytidine, deoxyuridine, cytosine, uracil, and thymine were added to the treated cultures in place of TDR at two- or four-times higher concentrations than BUDR. None of them, however, was found to be effective.

Gradual recovery from the inhibited state of HA synthesis produced by BUDR following transfer into the analog-free medium

This experiment was undertaken to examine whether the effect of BUDR was reversible or irreversible. As seen in figure 3, in control cultures an extremely constant rate of the mucopolysaccharide synthesis (26.7 $\mu\text{g}/10^6$ cells on the average) was observed throughout five successive transfer generations. In contrast, the cells grown in the medium containing BUDR at 5 $\mu\text{g/ml}$ for three days accumulated only

TABLE 1
Prevention of the inhibitory effect of BUDR by the simultaneous addition of TDR. 2.5×10^5 cells/dish were grown for three days in different media containing either BUDR alone, TDR alone, or both, at the designated concentrations

BUDR ($\mu\text{g/ml}$)	TDR ($\mu\text{g/ml}$)	Total no. of cells ($\times 10^{-6}$ /dish)	HA produced ($\mu\text{g}/\text{dish}$)	HA produced per 10^6 cells (μg)
—	—	2.84	69.2	24.4
5	—	2.15	27.2	12.6
5	5	2.30	33.0	14.3
5	10	2.94	61.2	20.8
5	20	2.87	65.5	22.8
—	20	2.92	69.2	23.7

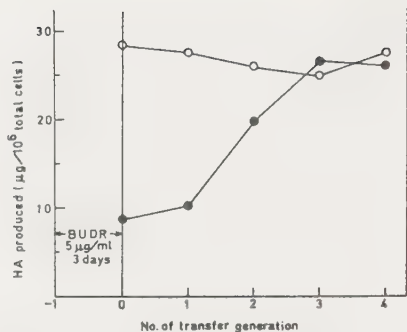


Fig. 3 Recovery from the inhibited state of HA synthesis produced by BUDR, following transfer into the analog-free medium. Two sets of cultures containing 2.5×10^5 cells/dish were incubated in the standard medium with and without $5 \mu\text{g}$ BUDR/ml for three days. The untreated cells were then subcultured every three days at the same inoculum size. The BUDR-treated cells were washed twice with the agent-free medium, transferred into the standard medium, and subcultured in the same manner as in the case of untreated cells. In each of the transfer generations, cell count and determination of the HA content were carried out. $\circ$ — $\circ$, control cultures; $\bullet$ — $\bullet$, BUDR-treated cultures.

about one-third ($8.6 \mu\text{g}/10^6$ cells) the amount of HA as in the control cultures. When these inhibited cells were harvested, washed twice with analog-free medium, transferred into fresh standard medium and subcultured every three days, a gradual but eventually complete recovery from the inhibited state of HA synthesis occurred. There was a lag period of recovery during one transfer generation (for 3 days) just after transfer into the analog-free medium, but then during the next two generations (for 6 days), the recovery was completely up to the control level. Thus, the effect of BUDR was clearly demonstrated to be reversible.

DISCUSSION

The present data indicate that in a clonal hybrid line, B-6, derived from the mating of mouse and Chinese hamster cell lines, HA synthesis is significantly inhibited by BUDR at relatively low concentrations which do not interfere very much with cell growth. This phenomenon is con-

sistent with the description by Bischoff and Holtzer ('68) of synthesis of HA in chick embryo amnion cells. Similar effects of the analog on some other differentiated functions such as myogenesis, chondrogenesis, and the synthesis of melanin have also been reported, and in these cases more detailed analysis has been carried out.

In the present experiments, low levels of BUDR ($1 \mu\text{g}/\text{ml}$ or less) did not suppress the growth of B-6 cells at all. However, the inhibitory effect on the synthesis of HA was significant and reproducible (as much as 40% decrease). At higher concentrations (5 – $25 \mu\text{g}/\text{ml}$), the analog inhibited the function by more than 70% as compared with the untreated controls, though some drop of the growth rate was detected. It is interesting to note that the extent of inhibition of cell multiplication and HA production is almost the same over the wide range of the drug concentrations from 5 to $25 \mu\text{g}/\text{ml}$. Bischoff and Holtzer ('68) have reported that proliferation of chick embryo amnion cells occurs normally even in as high as $50 \mu\text{g}/\text{ml}$ of BUDR, and that their HA synthesis is blocked completely. In our system we have not obtained as yet experimental conditions under which the drug inhibits exclusively the mucopolysaccharide production without any suppression of growth.

Kinetic study revealed that inhibition was detectable after two days of culture of the cells in the presence of the analog. This result, however, suggests the incubation time necessary to detect a significant difference between the HA contents in treated and untreated cultures, but does not necessarily represent the minimum time of treatment for the effectiveness of BUDR. Since B-6 cells propagate with a generation time of about 16 hours (Koyama and Ono, '70), they divide three times in two days. Recently Coleman et al. ('70) and Bischoff and Holtzer ('70) have reported that treatment with BUDR for less than one generation time, and even for one s period, is sufficient for suppression of chondrogenesis in chick cartilage, or of myogenesis in chick muscle cells.

Inhibition of HA synthesis was quite reversible after at least three days of incubation in the analog, in agreement with

the results reported for muscle (Stockdale et al., '64; Coleman et al., '69), cartilage (Abbott and Holtzer, '68; Lasher and Cahn, '69), pigmented retina (Coleman et al., '70) and melanoma (Silagi and Bruce, '70). In the present studies, being different from those in other systems, we could examine quantitatively the recovery with time after transfer of treated cells to the analog-free medium. The results obtained show that the effect of BUDR is long acting for a nine-day period, or more than ten generations.

Addition of TDR simultaneously with BUDR at two to four fold concentrations prevented the suppression of HA production. In contrast, none of other pyrimidines or pyrimidine nucleosides checked was effective. Lasher and Cahn ('69) have reported that uridine successfully counteracts the BUDR effect on chondrogenesis. This was not, however, the case in our cultures. Similar data have been described by Bischoff and Holtzer ('70) and Coleman et al. ('70).

BUDR is known to be incorporated into the DNA of a variety of cells including muscle and cartilage cells (Stockdale et al., '64; Abbott and Holtzer, '68) in place of thymidine. We have also obtained the same preliminary result in the cells used here by exposure to ³H-BUDR.

Some lines of evidence mentioned above support the hypothesis that BUDR suppresses HA synthesis by virtue of its incorporation into the DNA of B-6 cells. Bischoff and Holtzer ('70) have proposed to explain the inhibition of myogenesis fusion by this mechanism. In addition, this may be suggested by the finding that inhibition of DNA synthesis during exposure to BUDR prevents the suppression of cartilage formation (Coleman et al., '70). At present, whether the above hypothesis is true in our cultures or not remains undetermined.

The effects of excess TDR and FUDR are also interesting. Both are well known to inhibit DNA synthesis in mammalian cells. In fact, the growth of B-6 cells is blocked by the presence of either agent, but it should be emphasized that the treated cells continued to synthesize HA at a significant rate so that the amount of the mucopolysaccharide per cell in-

creased more than two-fold over the control for a three-day period. In our preceding paper (Koyama and Ono, '70), we postulated the existence of some kind of "shut-off mechanism" for HA synthesis which starts to operate with the cessation of cell growth, since actively growing cells are able to produce the mucopolysaccharide while completely stopping this activity in the stationary phase. In addition, hydroxyurea, another inhibitor of DNA synthesis, was reported to show a similar effect as that of the agents mentioned above. It is therefore evident that treatment of B-6 cells with all inhibitors of DNA synthesis disturbs the shut-off mechanism so that treated cells continue to synthesize HA in spite of the absence of cell multiplication.

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Note added in proof: Recently we have known the fact that the activity of HA synthetase involved in the synthesis of the mucopolysaccharide is reduced by more than 60% in the B-6 cells treated with BUDR at 5-25 $\mu\text{g/ml}$ for three days.

Immunoglobulin G and Free Kappa-Chain Synthesis in Different Clones of a Hybrid Cell Line

BEHZAD MOHIT

Cellular differentiation may be thought of as an orderly and tightly controlled process of selective gene expression. Recent experiments support this notion by demonstrating that large numbers of unexpressed genes are present in the nucleus of the differentiated cell (1-3). One approach to the study of the biochemical control mechanisms in cellular differentiation has been hybridization of cells that differ in their expression of specific genes. Such hybridization studies, however, have been complicated because of various degrees of heterogeneity in chromosome number present in the parental cell lines and also because of chromosome losses from the resulting hybrid cells upon successive cell divisions.

I have attempted to circumvent these difficulties by carefully choosing homogeneous parental cell lines for hybridization, and by subsequent cloning of the resulting hybrid cells. A hybrid cell line was established by cell fusion between a cloned BALB/c myeloma that produced immunoglobulin IgG and free kappa chain, and C₅₇BL/6n lymphoma that did not produce any immunoglobulins. The resulting hybrid cell population synthesized free kappa chain, but no synthesis of IgG (γ_{2a}) heavy chain was detected (4). I now report isolation of IgG (γ_{2a}) heavy-chain producers from these hybrid cells by a single-cell cloning technique. It will be shown that whether a hybrid cell is producing IgG (γ_{2a}) heavy chain and free kappa chain or only free kappa chain may be related to the chromosomal composition of the cell.

MATERIALS AND METHODS

Tissue Culture Conditions and Media. Cells were grown in Eagle's minimal essential (suspension) medium, supplemented with 400 mM glutamine, 1 ml of 100-times concentrated non-essential amino acids, and 1 ml of 100 mM sodium pyruvate (Microbiological Associates, Inc.); 100 units of penicillin and 20 ml of fetal calf serum per 100 ml of Eagle's medium (5).

Production of Hybrid Cells. Hybridization was performed as previously described by the use of Sendai virus inactivated with β -propiolactone. Growing hybrid cells were selected for in HAT medium (4).

Cloning Technique. Cloning was done by a modification of a technique described by Puck (6). Glass rings of 6-mm height and 7-mm internal diameter were cut from glass tubing. After thorough washing, they were soaked in saline at 37°C for a minimum of 30 min, rinsed three times with deionized-glass distilled water, and dried. Then, the rings were rimmed with nontoxic high-vacuum silicone grease (Dow Corning) and autoclaved. 10–30 cells of a monodisperse suspension in 3 ml of medium were added to 60-mm tissue culture dishes (Falcon plastics). After 2-hr incubation at 37°C in a CO₂ incubator, the attached single cells were visualized by phase microscopy and isolated within a glass ring. The silicone grease helped to seal off the isolated cell from the remainder of the cells in the plate. Cell division within the glass rings was followed each day and the medium within the ring was changed every 4 days. When the number of cells reached 500 or more within a ring, the cells were gently removed with a rubber policeman and subcultured. The decanted medium from each ring was tested for secreted immunoglobulins. To assure that there was no leakage in the silicone grease seal that might have allowed cells from outside the ring to enter into the cylinder, concentrated trypan blue dye or bacteria were added to the medium surrounding the glass ring 18 hr before subculturing. Dye or bacteria seldom entered a ring; most rings were perfectly sealed.

Other Procedures. Histocompatibility-2 (H-2) antigens present on the cells were determined according to Rogentine (7) by measurement of ⁵¹Cr release from labeled cells in the presence of specific H-2 antisera (Table 2) and rabbit complement. Chromosome preparations were made by a modification of the method described by Moorheat *et al.* (8), and chromosome numbers for each cell line were determined by counting

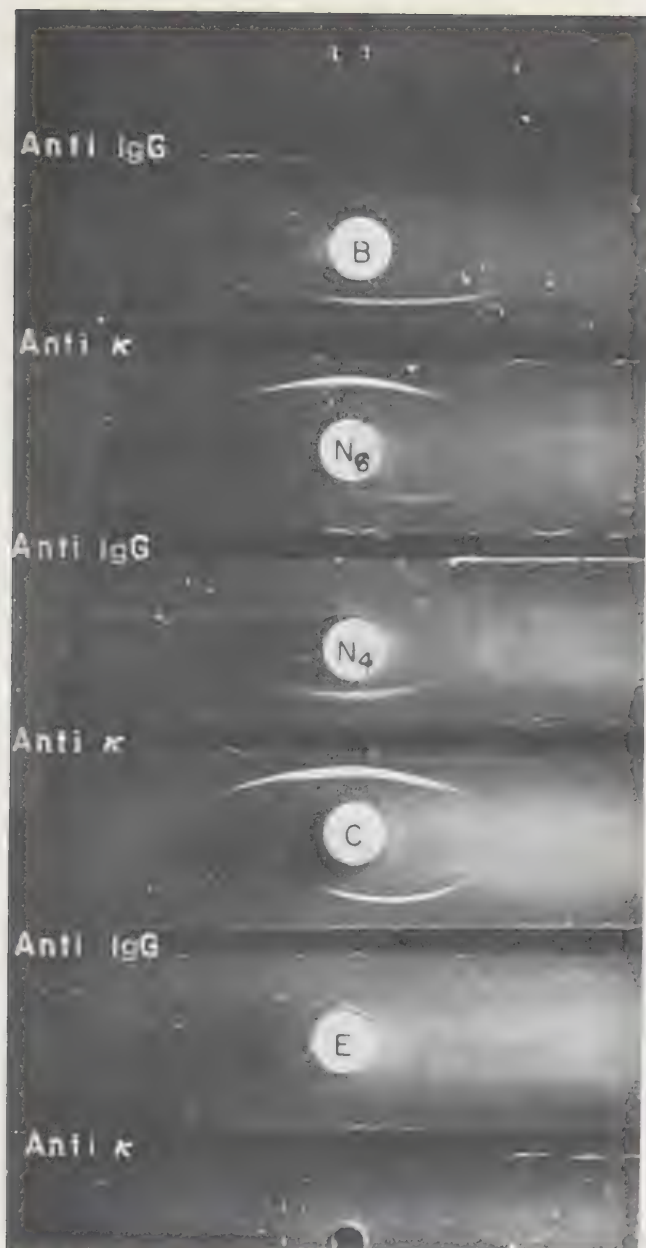


FIG. 1. Immunoelectrophoresis was done on proteins secreted by 2-day cultures of 1×10^7 cells concentrated to 0.2 ml (100 times) B = BALB/c serum; N_6 = N_6 hybrid; N_4 = N_4 hybrid; C = CL₄ (azaguanine-resistant) parent; E = EL₄ BrdU parent. Anti IgG and anti-kappa chain (anti κ) sera were monospecific for mouse IgG and kappa chains (4).

the chromosomes in photomicrographs of 25 randomly chosen metaphase cells. Immunoglobulins were determined by Ouchterlony gel diffusion and immunoelectrophoresis by use of monospecific antisera (4).

RESULTS

Characteristics of the parental cell lines and the mixed hybrid cell population

The BALB/c myeloma parent was established from a RPC-5 tumor generously provided by Dr. Michael Potter. It was subsequently cloned. One of the clones (CL₄) synthesized IgG and free kappa chain, and had a modal chromosome number of 61 that was stable in culture for 2.5 years. At this time, it was made resistant to 15.2 μ g/ml of 8-azaguanine (4). The 8-azaguanine-resistant subline continued to synthesize IgG and free kappa chain and had a modal chromosome number of 60 (Table 1). It was used as one of the parents for hybridization. The other parent was the bromodeoxyuridine-resistant EL₄ subline. This cell line was established from a lymphoma induced in a C₅₇BL/6n mouse by 9,10-dimethyl-1,2-benzanthracene (9). It had a diploid modal chromosome number [40] with a narrow range, therefore, it was made resistant to 100 μ g of bromodeoxyuridine per ml, without cloning. This bromodeoxyuridine-resistant subline had a modal chromosome number of 39 and did not produce any immunoglobulins (Table 1). The EL₄ $\times$ CL₄ hybrid cell population had a wide range of chromosome numbers [70–103]. Only kappa chain could be detected in this hybrid population. No intracellular or secreted IgG was detected (ref. 4, Table 1).

Growth in mice

The 8-azaguanine-resistant myeloma cells could easily be grown in BALB/c mice by injecting 5×10^5 or more cells subcutaneously. It continued to produce IgG and free kappa chain in the mouse. The bromodeoxyuridine-resistant lymphoma cells would easily grow in C₅₇BL/6n mice as subcutaneous or intraperitoneal tumors. The hybrid cell population did not grow in BALB/c or C₅₇BL/6n mice when 1×10^7 cells were injected subcutaneously, but grew in BALB/c $\times$ C₅₇BL/6n F₁ hybrids. Its growth rate was about one half that of the parental cell line. It produced Bence-Jones kappa chains that were excreted in the mouse urine, but no myeloma IgG was detected in the serum of the mice that bore tumors.

Characteristics of isolated clones of hybrids

Two groups of clones were isolated from the mixed hybrid cell population. Seven clones (M₁–M₇) were isolated from the hybrid cells that were grown in HAT medium for 3 months,

TABLE 1. Comparison of karyotypic composition and immunoglobulin synthesis of various clones isolated from the hybrid cell population

Cell lines*	Chromosomes			Intracellular and secreted immunoglobulins	
	Mode	Mean	Range	Markers	Kappa chain IgG
CL ₄ (azaguanine-resistant)	61	63.0	61-67	2m or 3m	+
EL ₄ (bromodeoxyuridine-resistant)	60 40	61.0 40.5	60-64 39-42	2m or 3m† 1s§	+
EL ₄ × CL ₄ † (mixed hybrid)	39	39.5	38-41	1s	-
N ₃	92	89.7	70-103	2m; 1s	+
N ₆	88	89.1	90-86	2m; 1s	-
N ₅	90	91.3	90-94	2m; 1s	+
N ₁	90	91.4	90-94	2m; 1s	+
N ₂	91	91.6	90-94	2m; 1s	+
N ₄	94	91.8	89-94	3m; 1s	+
N ₇	94	92.7	90-95	3m; 1s	+
N ₅	93	93.5	91-98	4m; 1s	+
N ₄	86	86.6	84-89	2m; 1s	+
N ₁	90	90.7	89-93	2m	+
N ₂	90	91.4	90-94	2s; 1m	+
N ₃	95	92.7	86-96	2m	+
N ₆	94	98.0	90-97	2m; 1s	+
N ₆	100	100.7	99-103	2m; 1s	+

* CL₄ is the cloned BALB/c myeloma parent; EL₄ is the diploid C₃H/6b lymphoma parent. M₁, M₂ are clones isolated from the hybrid cell population, grown in HAT for 3 months, passaged through mice twice, and maintained in HAT for four more months. N₁-N₆ are clones isolated from hybrid cells grown in HAT for 3 months and frozen in liquid nitrogen for 5 months.

† The modal chromosome numbers of CL₄ and azaguanine-resistant cells were given as 57 and 54, respectively; while the range for chromosomes of EL × CL₄ hybrid cells was given as 70-97 in a preliminary report (4). This underestimation of chromosome numbers was the result of errors of direct counting of chromosomes under the microscope. The numbers given here were obtained by counting the chromosomes in 25 photomicrographs of randomly selected metaphase cells. Secreted immunoglobulins were detected by Ouchterlony gel diffusion and immunoelectrophoresis of dialyzed and lyophilized medium from 1 × 10⁶ cells. Intracellular immunoglobulins were determined similarly in lysates of 4 × 10⁶ cells/ml. IgG present in clones N₃ and N₆ was precipitated with RPEC-5 (i.e., BALB/c myeloma) idiotypically specific antisera.

† m = metacentric.

§ s = submetacentric.

TABLE 2. *Comparison of H-2 antigens of parental cell lines and hybrid clones*

Alloanti- serum*	H-2 specificity	CL ₄ (Azaguanine- resistant)	EL ₄ (Bromo deoxy- uridine- resistant)	Hybrid- clones	
		parent	parent	N ₁ -N ₆	M ₁ -M ₇
C ₂	2(C ₅₇ BL/6n	0†	+‡	+	+
C ₈	8,9(BALB/c)	+	0	+	+

* The antisera were obtained from Dr. George Snell (PH 66-483), under contract with Transplantation and Immunology Branch, NIAID, NIH, Bethesda, Md. The nomenclature of antisera and their specificity are taken from the catalog of mouse alloantisera compiled by George Snell, 1968.

† 0 = antigen absent.

‡ + = antigen present (lysis at least 15% higher than control).

§ See Table 1, legend.

passed through mice for two generations, and subsequently maintained in HAT medium for 4 additional months. Six other clones (N₁-N₆) were isolated from the hybrid cells that were grown in HAT medium for 3 months and then frozen in liquid nitrogen for 5 months. M₁-M₇ and 4 of the N series of clones produced only free kappa chains. Clones N₃ and N₆, however, synthesized easily detectable amounts of IgG, as well as free kappa chains (Fig. 1, Table 1). The IgG heavy chains synthesized by these clones (kindly tested by Miss R. Lieberman) were of the BALB/c myeloma type, as shown by Ouchterlony gel diffusion with idiotypically specific antisera. Media from 2-day cultures of 1×10^7 cells of clones N₃ and N₆ were concentrated to 0.2 ml (100 times) and tested for the presence of C₅₇BL/6n-type IgG. The assay, kindly performed in Dr. L. A. Herzenberg's laboratory, consisted of inhibition of precipitation of radioactive C₅₇BL/6n IgG by allospecific antiserum. This assay could have detected as little as 3 μ g of C₅₇BL/6n IgG per ml. None was detected. The same samples contained about 1 mg/ml of BALB/c-type IgG.

It was particularly interesting that clones N₃ and N₆, which synthesized both IgG and free kappa chains, had the highest mean chromosome-number. Marker chromosomes of both parental cell lines were found in all the hybrid clones.

H-2 antigens of both parental cell lines were demonstrated in IgG-producing as well as kappa-producing clones.

DISCUSSION

The data presented provide evidence that immunoglobulin synthesis is maintained in hybrid cells derived from cloned immunoglobulin-producing mouse myeloma cells and non-producing mouse lymphoma cells. Most of the hybrid clones synthesize and secrete detectable quantities of kappa light chains only. Some clones, however, synthesize and secrete both IgG (γ_2a) and free kappa chain. The IgG heavy chain synthesized is of BALB/c (myeloma) specificity. No C₅₇-BL/6n (lymphoma)-type heavy chain is detected. Furthermore, those clones that synthesize both IgG and kappa chain have the highest mean chromosome number (Table 1).

These observations may be interpreted as follows: (a) IgG heavy-chain or kappa light-chain synthesis in these hybrid cells may be directly attributable to the presence in the cells of the myeloma chromosome(s) that carry the gene(s) for these polypeptide chains; (b) in the mouse, the genes for IgG heavy chain and kappa light chain are probably located on different chromosomes. This assertion is consistent with the observation that the hybrid clones that synthesize both IgG and kappa have higher chromosome numbers than those that synthesize only kappa chain. It is known that, in the rabbit, the genes for light and heavy chains of immunoglobulins are not linked (10, 11); (c) synthesis of IgG heavy chain in the hybrid cell is not the simple consequence of the presence of "gene activators" or other positive-control factors contributed by the CL₄-8-azaguanine-resistant parent. If this were true, one would have expected the silent IgG genes of C₅₇BL/6n type, which were presumably present in the IgG-synthesizing hybrid cells, to have been activated. However, no C₅₇BL/6n-type IgG heavy chain was detected in such hybrids; (d) detection of IgG heavy chain in hybrids with the highest chromosome number provides a strong argument against the notion that gene repressors or other negative-control molecules could have caused the absence of immunoglobulin synthesis in an EL₄ (bromodeoxyuridine-resistant) parent. If such negative-control factors were operative in this cell line, one would have expected both IgG synthesis and kappa light-chain synthesis to stop in hybrids with the highest chromosome number. Such hybrids, presumably, would have contained the EL₄ (bromodeoxyuridine-resistant) cell chromosomes for the "repressors".

These conclusions, however, may be challenged by several arguments.

(i) It was shown that the EL₄ (bromodeoxyuridine-resistant) parent had one chromosome less than the diploid number for the mouse. It is conceivable that the genes for both kappa light chain and IgG heavy chain, as well as the "repressor" genes for these polypeptides, were on that one chro-

mosome. This possibility, in my view, is unlikely.

(ii) Specific immunoglobulin gene "repressors" or "activators" may, indeed, exist but show allotypic specificity. Then, it would not be surprising that their presence could not be demonstrated in hybrids derived from cells of two different allotypic backgrounds. This argument derives further strength when it is recalled that the individual immunoglobulin-producing cells in a heterozygous animal synthesize immunoglobulin of one allotype only, a phenomenon known as "allelic exclusion" (12,13). In this regard, it is of interest to recall that hybrid clones of a cross between myeloma MOPC 315 of BALB/c origin that produced IgA and fibroblasts CL1D of a C₃H/He mouse produced little or no immunoglobulin (14). This is entirely expected if repressors for immunoglobulin synthesis require allotypic specificity, because it is known that IgA of BALB/c and C₃H/He mice are of the same allotype (15). The answer to this argument must await the results of studies on hybrids between two different immunoglobulin-producing myelomas of BALB/c mice. The interpretation of such studies, however, may be complicated by similar arguments invoking this time the idiotypic specificity of the control molecules involved in gene expression.

(iii) Finally, lack of repression of immunoglobulin synthesis in the present hybrid cells may reflect a mutation(s) at the "operator-gene" region of the immunoglobulin operon of CL₄-8-azaguanine-resistant myeloma parent. Such a mutation could have rendered the immunoglobulin operator gene(s) of the myeloma incapable of perfect binding of functional repressor that may have been contributed to the hybrid cells by the nonproducing EL₄ bromodeoxyuridine-resistant parent. Examples of "operator gene" mutants incapable of binding normal functional repressor are well documented for the *lac*-operon of *Escherichia coli* (16). Such mutants synthesize large amounts of *lac*-operon gene products in the presence of normal repressor (17). The possibility of occurrence of such an "operator-gene" mutation in a functional tumor such as CL₄-8 azaguanine-resistant myeloma may not be altogether remote. It is known that cell division and immunoglobulin synthesis are closely linked functions in normal lymphoid cells (18, 19). Therefore, if it is assumed that the processes of cell division and immunoglobulin synthesis in normal lymphoid cells are controlled by the same "operator gene", a single mutation at this gene locus could account for the emergence of immunoglobulin-synthesizing tumors. Elucidation of this intriguing possibility must await the development of inducible immunoglobulin-producing cell lines or the isolation of the hypothetical immunoglobulin gene repressors. In any event, it is difficult to imagine that "operator gene" mutations could account for the orderly process of selective

gene expression seen in cellular differentiation, even though such mutations may be the basis of the emergence of differentiated tumors such as the myeloma.

Several examples of loss of differentiated function in somatic hybrids have been reported. These include extinction of melanin synthesis in melanoma-fibroblast hybrids (20) and of secretion of growth hormone in pituitary-fibroblast hybrids (21). There are also examples of differentiated functions such as synthesis of hyaluronic acid and collagen that have continued when producer and nonproducer cells were hybridized (22). It is possible to explain the expression or lack of expression of a specialized gene in these hybrids by either the presence or absence of the chromosome carrying the structural gene or the gene specifying the repressor. In addition, various "operator genes" may have different degrees of specificity requirements for interacting with repressors. For example, the specificity requirement for immunoglobulin genes may be more stringent. Notwithstanding these arguments, an important point emerging from the present investigation is the need for caution in attributing gene "turn on" or "turn off", and thus deducing the presence of hypothetical control molecules in a mixed hybrid-cell population derived from heterogeneous parental cell lines. Closer attention to the karyotypic composition and homogeneity of the parental and hybrid cells to be studied are needed before conclusions regarding cellular control mechanisms are drawn.

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**The Use of Cell Hybridization
in the Study of Malignancy**

THE ANALYSIS OF MALIGNANCY BY CELL FUSION

I. HYBRIDS BETWEEN TUMOUR CELLS AND L CELL DERIVATIVES

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AND H. HARRIS

INTRODUCTION

We define malignancy as the ability of tumour cells to grow progressively and kill their host. The idea of using cell fusion to facilitate genetic analysis of this phenomenon is an attractive one; but there have so far been few studies of this kind. Barski, Sorieul and Cornefert (Barski, Sorieul & Cornefert, 1961; Barski & Cornefert, 1962) examined hybrid cells that arose spontaneously in mixed cultures of two mouse cell lines, one highly malignant, the other less so. The ability of the hybrid cells to produce tumours resembled that of the more malignant parent. Hybrids between the more malignant of these two parent cells and normal mouse fibroblasts also appeared to be malignant (Scaletta & Ephrussi, 1965); and so did the hybrids that arose spontaneously in mixed cultures of non-malignant mouse cells and mouse cells rendered malignant by infection with polyoma virus (Defendi, Ephrussi, Koprowski & Yoshida, 1967). These latter hybrids continued to produce the T antigen characteristically associated with polyoma virus infection (Defendi *et al.* 1967; Defendi, Ephrussi & Koprowski, 1964). The

conclusion drawn from these studies was that malignancy was a dominant character in somatic cell hybrids and that therefore the lesion resulting in malignant behaviour was unlikely to involve a loss of genetic information. Silagi (1967) studied the behaviour of hybrids between cells of a malignant melanoma and A9 cells (a mouse fibroblast line lacking the enzyme inosinic acid pyrophosphorylase). The *in vivo* behaviour of these hybrids was less clear cut. Some clones produced tumours in a majority of the animals injected; others produced few tumours or none at all. In all of these studies relatively large inocula of cells were used and little attention appears to have been paid to the possibility that the tumours resulting from the injection of the hybrid cells might have arisen from variants in the hybrid cell population that did not reflect the overall level of malignancy of the population as a whole. Since injection of cells into the animal selects for malignancy, the conclusion that malignancy is a dominant characteristic in hybrid cells must remain precarious unless one can exclude the possibility that the tumours produced result from the selective outgrowth of a minor subpopulation of the hybrid cells injected. This hazard applies, of course, to any cell population injected into the animal; but it is especially serious in the case of somatic cell hybrids, because these hybrids are known to exhibit progressive loss of chromosomes and other forms of chromosomal instability (for review, see Harris, 1970). These cells therefore present an unusually high degree of genetic variation on which selection could operate. Barski & Cornefert (1962) and Ruddie, Chen, Shows & Silagi (1970) have examined the chromosomal constitution of some cell populations explanted from tumours produced by the injection of hybrid cells; but, with the possible exception of a single tumour examined by Silagi (1967), no information appears to have been published about the chromosomal constitution of the tumours themselves. It is therefore difficult to assess the extent to which previous studies indicating a dominance of malignancy in somatic cell hybrids might have been complicated by the loss of chromosomes or by selection of atypical variants *in vivo*. The introduction of the Sendai virus cell fusion technique (Okada, 1958; Harris & Watkins, 1965) has made it possible to hybridize virtually any mammalian cells and has thus greatly increased the range and power of this form of analysis. It therefore seemed worthwhile, in view of the great biological and clinical importance of malignancy, to re-investigate these questions in a more systematic way. The present series of papers deal with the growth *in vivo* of hybrids produced by fusing a range of highly malignant mouse cells with cells of established lines of low tumorigenicity and with diploid cells isolated directly from the animal. Preliminary accounts of some of this work have appeared previously (Harris *et al.* 1969; Harris & Klein, 1969); and a detailed analysis of the immunological characteristics of the hybrid cells and the tumours arising from them has been published elsewhere (Klein, Gars & Harris, 1970).

MATERIALS AND METHODS

The tumours

Cells of the following ascites tumours were used: Ehrlich, SEWA, MSWBS, YAC and YACIR. The Ehrlich tumour was originally derived at about the turn of the century from a mammary carcinoma in a mouse of unknown genetic constitution. This tumour has been passaged continuously in mice of different genotypes and appears to have evolved mechanisms that greatly

reduce the expression of its histocompatibility antigens (Hauschka & Amos, 1957). It grows in any strain of mouse and produces lethal neoplasms when injected subcutaneously as well as intraperitoneally: a few cells injected into the peritoneal cavity will kill most mice within 3 weeks; even one cell is lethal in about 15 % of recipient animals (Hauschka, 1953). The SEWA tumour is an ascites sarcoma originally induced in 1960 by the injection of polyoma virus subcutaneously into a newborn *A.SW* mouse (Sjögren, Hellström & Klein, 1961). This tumour carries the H-2^s histocompatibility antigen complex and its growth is limited to mice of the *A.SW* strain. It also carries the polyoma-specific transplantation antigen. Although originally an osteogenic sarcoma, the tumour now shows no osteogenic differentiation. An inoculum of 1000 cells produces neoplasms in 100 % of genetically compatible unirradiated mice, and an inoculum of 100 cells produces neoplasms in a 100 % of mice given 400 rd (4 J kg⁻¹) of total body X-irradiation (Sjögren, 1964). The SEWA tumour was converted to the ascites form in 1968 (Nordenskjöld, 1968), and, in this form, an inoculum of 10⁶ cells kills most animals in 3–4 weeks. Like SEWA, the MSWBS tumour is an ascites sarcoma. It also carries the H-2^s histocompatibility antigen complex and is specific for the *A.SW* strain of mice. It was originally derived from a tumour induced by the injection of methylcholanthrene into the thigh muscle of a male *A.SW* × *A.F*₁ hybrid mouse (Klein & Klein, 1958). The variant used in the present experiments arose from cells passaged in the parental *A.SW* strain. This variant has lost the H-2^a antigen complex derived from the A strain parent, but has retained the H-2^s complex of the *A.SW* strain parent. Growth of the tumour is restricted to *A.SW* mice. An inoculum of as few as 50 cells produces neoplasms in 100 % of unirradiated genetically compatible mice. The neoplasms are lethal within 14–20 days. The YAC and YACIR tumours are 2 different ascitic sublines of a lymphoma induced in a mouse of the *A/Sn* strain by Moloney virus (Klein, Klein & Houghton, 1966). The YACIR subline was derived from YAC by serial passage in strain A hosts immunized against the surface antigen associated with Moloney virus (Fenyö, Klein, Klein & Swiech, 1968). YACIR shows a 10-fold reduction in the concentration of the Moloney surface antigen, compared with YAC, and is resistant to the cytotoxic action of anti-Moloney antisera. With inocula of about 10 cells, both YAC and YACIR sublines produce neoplasms in 100 % of strain A mice, but the cells are rejected in other strains of mice. With inocula of 10 cells, the neoplasms produced are usually fatal within 10–14 days.

The cell lines

The established cell lines used (A9, B82 and A9RI) were all derivatives of the L cell (Earle, 1943). The A9 cell was obtained by progressive selection for resistance to 8-azaguanine (Littlefield, 1964*a*) and lacks the enzyme inosinic acid pyrophosphorylase, a defect that prevents its growth when *de novo* synthesis of purines is blocked by aminopterin (Littlefield, 1964*b*). The B82 cell was obtained by selection for resistance to bromodeoxyuridine and lacks the enzyme thymidine kinase (Littlefield, 1966). The A9RI cell line was derived from a single colony of cells that arose in bulk cultures of A9 cells exposed to HAT medium (see below). This medium prevents the growth of A9 cells, which lack inosinic acid pyrophosphorylase, but not of cells that possess this enzyme. This single colony of cells, which survived the treatment of the A9 cultures with HAT medium, proved on further subculture to be refractory to the inhibitory effects of this medium and to contain a high level of inosinic acid pyrophosphorylase. The inosinic acid pyrophosphorylase activity of the L cell is approximately 100 units per unit protein, that of the A9 cell is barely detectable and that of the A9RI cell is approximately 60 units per unit protein. Chromosomal analysis of the A9RI cell showed a karyotype essentially similar to that of the A9 cell, but the modal number of chromosomes was slightly lower than that of the wild type A9 cultures. The chromosomal constitution of the various parental cell lines is given in Table 1. The A9, B82 and A9RI cells all bore the H-2^k histocompatibility antigen complex. This is consistent with their derivation from L cells which were originally obtained from C3H mice carrying the H-2^k histocompatibility complex.

Conditions of cell culture and culture media

The cells were grown as monolayers in plastic flasks (Falcon Plastics, Los Angeles, California, U.S.A.) or glass bottles in Dulbecco's medium (Vogt & Dulbecco, 1963) or HAT medium. HAT medium was originally devised by Szybalska & Szybalski (1962) to select

against cells lacking inosinic acid pyrophosphorylase. It is composed essentially of Eagle's minimum essential medium (Eagle *et al.* 1956) with added aminopterin, hypoxanthine, thymidine and glycine at concentrations of 4×10^{-7} M, 1×10^{-4} M, 1.6×10^{-5} M and 3×10^{-6} M respectively. All media contained foetal calf serum at a concentration of 10% and the following antibiotics: penicillin 200 μ g, streptomycin 200 μ g, kanamycin 30 μ g, neomycin 30 μ g and mycostatin 40 μ g/ml.

Cell fusion

The cells were fused together by means of inactivated Sendai virus as described by Harris & Watkins (1965). The Ehrlich and SEWA cells fused readily with the L cell derivatives, but the MSWBS cells fused rather less well; the YAC and YACIR lymphomas fused poorly. Adequate numbers of heterokaryons could, however, be formed from the more recalcitrant cell types by increasing the concentration of Sendai virus and the relative input of the less fusable cell types (Harris, Watkins, Ford & Schoeff, 1966).

Selection of hybrid cells

Ehrlich, SEWA, YAC and YACIR cells adhere poorly to glass or plastic and may be eliminated simply by frequent changes of medium. MSWBS cells do adhere, but they are easily detached and they grow rather poorly on the plastic surface, at least initially. Selection against the tumour cells thus presented little difficulty. A9 and B82 cells do not grow in HAT medium, so that when these cells are fused with the tumour cells and the virus-treated cell population is grown in HAT medium, only hybrid cells, in which the tumour cells complement the enzymic defects of the A9 or B82 cells, survive.

Selection of hybrids between the Ehrlich cells and the A9RI cells presented a more difficult problem, since growth of the A9RI cells, which have a high level of inosinic acid pyrophosphorylase, is not inhibited by HAT medium. In this case, differences between the adhesive properties of the A9RI cells and the hybrids between these cells and the Ehrlich cells were exploited to select for the hybrids. Whereas the Ehrlich cells did not adhere to the plastic or glass surfaces of the culture vessels, the Ehrlich/A9RI hybrids did adhere to these surfaces, but much less firmly than the A9RI cells themselves. The hybrid cells were thus more easily detached from the floor of the flasks than the A9RI cells and they frequently came away spontaneously at mitosis. The cultures were therefore passaged by shaking the contents vigorously and transferring the resulting cell suspension to fresh flasks. This procedure led to progressive enrichment of the cultures with hybrid cells and, after several passages, to the production of pure cultures of hybrid cells.

Chromosomal analysis

Metaphase spreads of ascites tumours and of cells growing *in vitro* were made by the air drying technique of Rothfels & Siminovitch (1958). The cells were exposed to colcemid (Ciba Pharmaceutical Products Inc.) at a concentration of 0.1 μ g/ml for 1–2 h, treated with 0.9% sodium citrate solution for about 10 min and then fixed in 1:3 acetic acid-methanol. The fixed cells were spread on wet slides, air dried and stained with buffered Giemsa solution. Chromosome preparations from solid tumours were made in the following way. The mice bearing the tumours were given a dose of colcemid equivalent to 8–10 μ g/g of body weight and then killed 6–8 h later. Small pieces of the tumour were passed through a fine steel gauze mesh in 0.9% sodium citrate solution or in tissue culture medium diluted 1:5 with water. This procedure took 30–40 min. A fine suspension of cells was separated from the tissue mince by slow centrifugation and this cell suspension was then fixed and stained in the same way as the cells of the ascites tumours.

Assay for tumorigenicity

Except where one of the parents was the Ehrlich cell, all hybrids were assayed in syngeneic F_1 mice: the SEWA/A9 and MSWBS/A9 hybrids in $A.SW \times C_3H$, the YAC/A9 and YACIR/A9 hybrids in $A \times C_3H$. The tumour cells grow as well in the F_1 hybrid test animals as

in the parental strains. The original genotype of the mouse in which the Ehrlich tumour arose is not known, so that assays of the hybrids between the Ehrlich cells and the L cell derivatives were carried out in *C3H* mice, from which the L cell was derived. The Ehrlich tumour grows perfectly well in *C3H* mice, as in all other strains. In general, the histocompatibility antigen complexes of both the parent cells were expressed in the hybrids, although in hybrids in which one of the parents was an Ehrlich cell, the histocompatibility antigens of the other parent were expressed poorly (Klein *et al.* 1970). The possibility remained, however, that minor degrees of histoincompatibility might have been generated between the long established cell lines and the animals from which they were initially derived; and cell fusion might itself have given rise to new, undetected, antigenic combinations, even though direct tests on the hybrids showed the presence of both the expected parental antigen complexes. Assays for tumorigenicity were therefore carried out on newborn mice some of which were given 4 J kg^{-1} of whole body X-irradiation. Since newborn mice given this dose of radiation commonly support the growth of tumours bearing foreign H-2 antigens, it seemed unlikely that this assay would be complicated to any important degree by histoincompatibility between the hybrid cells and the test animals. In the event, this expectation was fulfilled. As will be seen later, when tests in syngeneic irradiated newborn mice revealed differences in tumorigenicity among the different clones of the one hybrid cell type, these differences were also apparent when the cells were inoculated into allogeneic irradiated newborn mice. In the tests the cells were injected subcutaneously in inocula of between 4×10^4 and 3.3×10^6 cells. Small cohorts of animals were injected from time to time, as cells and appropriate F_1 hosts became available. The inoculated animals were examined for development of tumours at weekly intervals. No animal was scored as negative until a period of 3 months had elapsed, and many groups of animals were observed for 5–6 months. In selected groups, growth of the tumours was recorded by regular caliper measurements: 3 diameters were measured and the geometric mean calculated.

Although some of the tumours produced in test animals by the injection of the hybrid cells could not be successfully passaged, others proved to be transplantable. These were carried in unirradiated adult mice syngeneic with the animal in which the tumour originally arose. Solid tumours were passaged by the subcutaneous injection of 0.2 ml of a crude cell suspension. Tumours converted to the ascites form were passaged by intraperitoneal injection of 0.2 ml of undiluted ascitic fluid. Occasional ascites tumours arose directly from the injection of the hybrid cells intraperitoneally into unirradiated adult mice.

Assay for inosinic acid pyrophosphorylase

This was carried out by P. R. Cook using the method described by Harris & Cook (1969).

RESULTS

Growth of the L cell derivatives in vivo

It will be seen from Table 2 that A9 cells and B82 cells have a very limited ability to grow progressively *in vivo* in irradiated newborn *C3H* mice. Even with very large inocula these cells rarely give rise to progressive tumours. The A9RI cell produces tumours more readily than the A9 cell; but it still has a very low level of tumorigenicity in comparison with the various tumours used in the present study.

Ehrlich/A9 hybrids

Clones of cells derived from these 2 parents showed great variation in cell shape, clonal morphology and growth rate. Tight epithelial clones, clones composed of long spindle-shaped cells growing in parallel, highly dispersed clones of irregular fibroblastic cells and a whole range of intermediate categories were observed. The *in vivo* tests were carried out on the most vigorous hybrids, which soon overgrew the bulk cultures. These hybrids grew rapidly *in vitro* with a generation time of between 10 and

12 h. The hybrid nature of the cells was confirmed by karyotypic analysis (Table 3). Both the Ehrlich cell and the A9 cell have specific chromosomal markers (Harris *et al.* 1965; Engel, McGee & Harris, 1969). The markers of both parent cells were identified in the hybrids. The hybrid cells initially contained a modal number of 128 chromosomes of which approximately 24 were bi-armed. This is very close to what one would expect from the fusion of one modal Ehrlich cell and one modal A9 cell. The cells showed little change in chromosome number during the first 2 months in culture, but, on more prolonged cultivation, chromosomes were progressively eliminated. As shown in Table 4, the Ehrlich/A9 hybrids had a very low level of tumorigenicity, comparable to that seen in the A9 cell. With inocula of up to 3.5×10^6 cells, the cumulative take incidence was only about 10%, even in irradiated newborn animals. Because these hybrids were selected in medium containing high concentrations of aminopterin, thymidine and hypoxanthine, their ability to produce tumours was also tested after the cells had been adapted to growth in medium not containing these additives. The take incidence was not increased. It is clear that the highly malignant character of the Ehrlich cell was not transmitted to the hybrid cell.

Tumours produced by Ehrlich/A9 hybrids

Although the tumorigenicity of these hybrids was very low, occasional solid and ascites tumours did develop after variable latent periods of 3–12 weeks. A few of these tumours proved to be transplantable. When, however, the chromosomes of these tumours were examined, the cells in the tumours were found to have a much lower modal chromosome number than that of the hybrid cells originally injected into the animal. Whereas the initial hybrid cell population had, at the time of injection, a modal chromosome number of about 128, the tumours produced had modal chromosome numbers in the eighties (Table 5). That the cells of which the tumours were composed were derived from hybrid cells was, however, clear from the presence in them of both Ehrlich and A9 chromosomal markers. The tumours were thus produced, not by the continued progressive growth *in vivo* of the hybrid cell population as a whole, but by selective overgrowth of variants which had lost chromosomes. This form of selection in the progressive growth of tumours is, of course, well known (for review see Berger, 1969). The results thus appeared to indicate that Ehrlich/A9 hybrids containing the complete chromosomal complements of both parents showed little or no capacity for progressive growth *in vivo*, for even when the injection of such hybrids did produce occasional tumours, these were not composed of cells with unreduced chromosome complements.

These results raise several obvious questions. (1) Is the suppression of malignancy by cell fusion limited to the Ehrlich ascites tumour? (2) Is the effect peculiar to the A9 cell? (3) Does the metabolic defect in the A9 cell (inosinic acid pyrophosphorylase deficiency) play an important role in the suppression of malignancy? (4) Can the suppressive effect be exercised by normal diploid cells? (5) Is it, in general, the case that when a malignant cell and a non-malignant cell are fused together, the hybrids with unreduced chromosome complements are not malignant? (6) Is a loss of chromosomes essential for the production of malignant variants from a non-malignant hybrid

cell population? (7) If loss of chromosomes is essential, is it necessary to eliminate certain specific chromosomes, or is it enough simply to achieve some overall reduction in chromosome number? In the experiments now to be described attempts were made to investigate each of the above questions.

SEWA/A9, MSWBS/A9, YAC/A9 and YACIR/A9 hybrids

The hybrid nature of all these cell lines was confirmed by karyotypic analysis and by the presence on the surface of the cells of both the parental sets of H-2 antigens (Harris *et al.* 1969; Klein *et al.* 1970; unpublished observations by Eva-Maria Fenyő & Gertrud Grundner). The chromosome constitutions of the hybrid lines are shown in Table 3. The modal chromosome number of the MSWBS/A9 hybrid was initially almost exactly the sum of the modal chromosome numbers of the 2 parent cells. The modes of the SEWA/A9, YAC/A9 and YACIR/A9 hybrids were a little lower than those to be expected from the fusion of 2 modal parent cells. Marker chromosomes characteristic of the A9 cell were identified in all hybrids. SEWA and MSWBS markers were also identified in the SEWA/A9 and the MSWBS/A9 hybrids; but for the YAC/A9 and YACIR/A9 hybrids, karyological identification rested on the presence of the A9 markers, the total number of chromosomes and the proportion of bi-armed chromosomes, since the YAC and YACIR tumours lack specific chromosomal markers. The identification of these hybrids was, however, confirmed by the detection on the surface of the cells of the H-2 antigen complexes of both parents (observations by Eva-Maria Fenyő & Gertrud Grundner). The chromosomal constitutions of all these hybrids were initially very stable *in vitro*. There was little change in the modal chromosome number over the first weeks of cultivation, but, after several months, progressive loss of chromosomes was observed (Table 3). The stability of the karyotype in these hybrids *in vitro* resembled that previously described for a wide range of intraspecific hybrid cells (for review see Harris, 1970.)

The growth of these hybrids *in vivo* is shown in Table 4. It will be seen that in all cases the malignancy of the tumour cell was profoundly modified by fusion with the A9 cell. All these hybrids had a very low level of malignancy compared with that of the tumour cell parent. The take incidences of the SEWA/A9 and MSWBS/A9 hybrids were slightly higher than that of the Ehrlich/A9 hybrids; but the YAC/A9 and YACIR/A9 hybrids produced very few tumours. It is clear that the A9 cell has the ability to suppress the malignancy not only of the Ehrlich ascites cell, but also a wide range of other tumour cells.

The chromosomal constitutions of some of the tumours produced by the SEWA/A9 and MSWBS/A9 hybrids and of all the tumours produced by the YAC/A9 and YACIR/A9 hybrids were examined. In all cases the cells in the tumours showed a marked reduction in modal chromosome number relative to that of the hybrid cell population injected. As in the case of the Ehrlich/A9 hybrids, the tumours arose, not by progressive growth of the hybrid cell population as a whole, but by selective overgrowth of cells that had lost chromosomes.

The question arises whether the malignancy of these tumour cells might not be suppressed simply by continued cultivation *in vitro*. One subline of the MSWBS tumour

and 2 sublines of the SEWA tumour were therefore adapted to growth *in vitro* and cultivated continuously for several months under the same conditions as the hybrid cells. The ability of these tumour cells cultivated *in vitro* to grow progressively *in vivo* was then tested. The results of the tests are shown in Table 6. Prolonged cultivation *in vitro* has clearly not suppressed the malignancy of these tumour lines.

Ehrlich/B82 hybrids

In order to test whether the ability to suppress malignancy was limited to the A9 cell, hybrids were made between the Ehrlich cell and the B82 cell, an L cell derivative lacking thymidine kinase. These hybrids initially had a slightly lower modal chromosome number than the Ehrlich/A9 hybrids (Table 3). The take incidence for the Ehrlich/B82 hybrids is shown in Table 4: it is little different from that of the Ehrlich/A9 hybrids. The B82 cells are thus no less effective than the A9 cells in suppressing the malignancy of the Ehrlich tumour cells.

Ehrlich/A9RI hybrids

Both the A9 and the B82 cells are, however, cells with severe metabolic defects. Although the Ehrlich cells have a very high level of both inosinic acid pyrophosphorylase and thymidine kinase, it is possible that other metabolic derangements associated with inosinic acid pyrophosphorylase deficiency (Felix & DeMars, 1969), or thymidine kinase deficiency, might limit the growth of the hybrid cells *in vivo*. The Ehrlich cell was therefore fused with the A9RI cell, apparently a revertant of the A9 cell in which inosinic acid pyrophosphorylase activity is restored. (The inosinic acid pyrophosphorylase level in the A9RI cell is about 60 units per unit protein, compared with about 100 units for the L cell.) The take incidence for the Ehrlich/A9RI hybrids (Table 4) was indeed somewhat higher than that for the Ehrlich/A9 hybrids, but the level of tumorigenicity was still very low compared with that of the Ehrlich tumour cells. It was therefore clear that the ability of the A9 cell to suppress malignancy was not simply a consequence of its inosinic acid pyrophosphorylase deficiency, although it was possible, since the take incidence of the Ehrlich/A9RI hybrid was higher than that of the Ehrlich/A9 hybrid, that the inosinic acid pyrophosphorylase deficiency of the A9 cell might have been a contributory factor. It is, however, very unlikely that the growth of the hybrid cells *in vivo* is limited by low inosinic acid pyrophosphorylase levels. The take incidence of the Ehrlich/A9RI hybrids, despite the contribution from the Ehrlich cell, was actually lower than that of the A9RI cells themselves (Tables 2, 4); and analysis of some ascitic variants of transplantable tumours arising from Ehrlich/A9 hybrids showed no correlation between inosinic acid pyrophosphorylase content and growth rate.

Tumorigenicity of Ehrlich/A9 hybrids after prolonged cultivation in vitro

The most striking feature of the tumours produced by the hybrids tested in the present investigation was the gross reduction in the modal chromosome number of the tumour cells compared with that of the hybrid cells injected. This indicated that in these hybrid cell populations only cells that had lost chromosomes were capable of

progressive growth *in vivo*, or, at least, that the growth of such cells was very much more rapid *in vivo* than that of the unreduced hybrids. If the generation of malignant variants from an essentially non-malignant hybrid cell population required simply an overall reduction in chromosome number and not the loss of certain specific chromosomes, one might expect that the tumorigenicity of the hybrid cell population would increase if the cells were grown *in vitro* long enough to permit substantial chromosome loss to occur. The Ehrlich/A9 hybrids were therefore grown *in vitro* for a period of about 18 months during which the modal chromosome number dropped from about 128 to less than 80 (Table 3), the latter figure being comparable to the modes found in the various tumours derived from Ehrlich/A9 hybrids (Table 5). However, as shown in Table 4, the take incidence for these hybrids with grossly reduced chromosome numbers was not higher than that obtained before substantial loss of chromosomes had occurred. This finding indicates that the generation of malignant variants in the non-malignant hybrid cell population requires the loss of specific chromosomes and not simply an overall reduction in chromosome number. It appears that injection of the cells into the animal selects for just those hybrids that have lost the specific chromosomes responsible for suppression of the malignancy; but growth *in vitro* does not select for these hybrids, so that their incidence in the population is not greatly increased. Analysis of chromosome losses in intraspecific mouse cell hybrids cannot provide conclusive evidence for this interpretation, but the information that can be obtained is entirely consistent with it. After 18 months' cultivation *in vitro*, when the modal chromosome number of the Ehrlich/A9 hybrid cells had fallen from 128 to less than 80, about two-thirds of the bi-armed chromosomes derived from the A9 cell were retained; but in the tumours derived from these hybrids a comparable reduction in chromosome number was often associated with preferential elimination of the bi-armed A9 chromosomes, and in some tumours all but one or two of these chromosomes were eventually eliminated (Table 7). This does not, of course, mean that the chromosomes responsible for the suppression of malignancy were necessarily the bi-armed A9 chromosomes. Indeed, the studies of Ruddle *et al.* (1970) on tumours derived from hybrids between malignant melanoma cells and A9 cells suggest that this is not so. These tumours appeared to be composed of cells in which the bi-armed A9 chromosomes were retained, although the observations were subject to some uncertainty because the chromosomal analyses were not done on the tumours themselves, but on populations of cells explanted from the tumours and grown *in vitro* before analysis. It is, however, clear that the chromosomes eliminated from the cells that grow progressively *in vivo* are not the same as those lost on continued cultivation of the cells *in vitro*.

DISCUSSION

The present findings show that the ability of a wide range of different malignant cells to grow progressively *in vivo* can be suppressed when these cells are fused with certain derivatives of the L cell line. The highly malignant character of the tumour cells is suppressed whether or not the L cell derivatives have metabolic defects that

facilitate selection of the hybrid cells. So long as these hybrid cells retain the complete chromosome complements of the two parent cells, their ability to grow progressively *in vivo* is very limited, for tumours composed of such unreduced hybrids have not been found. However, when they lose certain specific, but as yet unidentified, chromosomes, the hybrid cells may regain the ability to grow progressively *in vivo* and give rise to a tumour. These results indicate that the L cell derivatives contribute something to the hybrid that suppresses the malignancy of the tumour cell, and this contribution is lost when certain specific chromosomes are eliminated. We have now to see whether this suppressive effect can also be produced by normal diploid cells and, if so, whether in this case also, reversion to malignancy is associated with loss of chromosomes.

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THE ANALYSIS OF MALIGNANCY BY CELL FUSION

II. HYBRIDS BETWEEN EHRlich CELLS AND NORMAL DIPLOID CELLS

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AND H. HARRIS

INTRODUCTION

In the present paper the characteristics of hybrids between Ehrlich ascites cells and normal diploid fibroblasts are described. Three wild type populations, each derived from different fusion experiments, and a number of clonal populations, each derived from a separate primary fusion, were examined.

MATERIALS AND METHODS

Cell types

The fibroblasts were obtained by trypsinization of 10-day-old embryos from *CBA* mice bearing the T6T6 chromosomal translocation (Ford, Hamerton, Barnes & Loutit, 1956). The primary cultures were grown for a few days in plastic flasks and then transferred by trypsinization to fresh flasks. The secondary cultures, and occasionally later subcultures, were used for the fusion experiments.

Cell fusion

A mixed suspension of 5×10^6 Ehrlich cells, taken directly from the mouse peritoneal cavity, and 5×10^6 fibroblasts, harvested from monolayers by trypsinization, were treated with 1000 haemagglutinating units of inactivated Sendai virus. The fused cells were introduced into plastic flasks and grown on the floor of the flasks in Dulbecco's medium.

Selection of hybrids

The Ehrlich cells, which did not adhere to the floor of the culture flasks, were eliminated by frequent changes of medium during the first few days. The Ehrlich/fibroblast hybrid cells adhered to the plastic surface much less tenaciously than the fibroblasts themselves and frequently came away from the floor of the flask at mitosis. When the cultures had become confluent, the flasks were shaken vigorously and the cells in suspension transferred to a fresh flask. Subcultivation by this procedure led to progressive enrichment of the cultures with hybrid cells and eventually to the production of pure cultures of hybrid cells.

In order to obtain clonal populations of hybrid cells, each derived from a separate primary fusion, the fused cells were trypsinized after 3 days' cultivation and the trypsinized cells seeded into 2-in. (5-cm) diameter plastic Petri dishes at a concentration of 50 cells per dish. The dishes were then incubated until visible clones appeared, usually a period of about 2 weeks. The clones were then examined microscopically and those composed of cells with a morphology clearly different from that of the fibroblasts themselves were selected for harvesting. Each selected clone was trypsinized into a small metal cylinder attached to the floor of the dish around the clone by a film of silicone grease, and the resulting cell suspension was introduced into a small plastic flask. Each clone was then grown up to give a population of cells large enough for karyotypic analysis and assay *in vivo*. The clonal populations were first assayed for growth *in vivo* just as soon as enough cells were available to provide an adequate inoculum; but subsequent assays were carried out on populations maintained by repeated subcultivation *in vitro*.

Assay for tumorigenicity

Most assays were carried out in newborn *CBA* mice given 400 rd (4 J kg^{-1}) of X-irradiation. A few tests were also made in unirradiated newborn *CBA* mice. Inocula of 10^4 to 2×10^6 cells were injected subcutaneously and the animals were scanned in the usual way for the appearance of tumours.

RESULTS

Growth of Ehrlich/fibroblast hybrids *in vivo*

All hybrid populations were identified by the presence of both the Ehrlich and the T6 chromosomal markers in at least some of the cells in each population. The results of the assays for growth *in vivo* are given in Table 1. It will be seen that all wild type and clonal populations were highly tumorigenic. Although there was some variation in take incidence from one clonal population to another, none of the clones showed a take incidence lower than 60%. There were, moreover, some differences in the growth rates of the tumours produced by different cell populations.

Chromosomal constitution of the hybrid cells

The high take incidence of all the Ehrlich/fibroblast hybrids would, at first glance, lead one to conclude that in this combination the malignancy of the Ehrlich cell was dominant. However, an examination of the chromosomal constitution of the hybrid cells revealed a situation of greater complexity. Tables 2 and 3 and Fig. 1 show the

chromosomal constitutions of the 3 wild type populations and of all the clonal populations. All of these analyses were done just as soon as populations large enough for the purpose could be obtained. The chromosome number to be expected in a hybrid cell resulting from the fusion of one modal Ehrlich cell and one fibroblast would be about 116; but it will be seen that none of the Ehrlich/fibroblast hybrids showed this modal chromosome number. Apart from populations composed of clearly polyploid clones

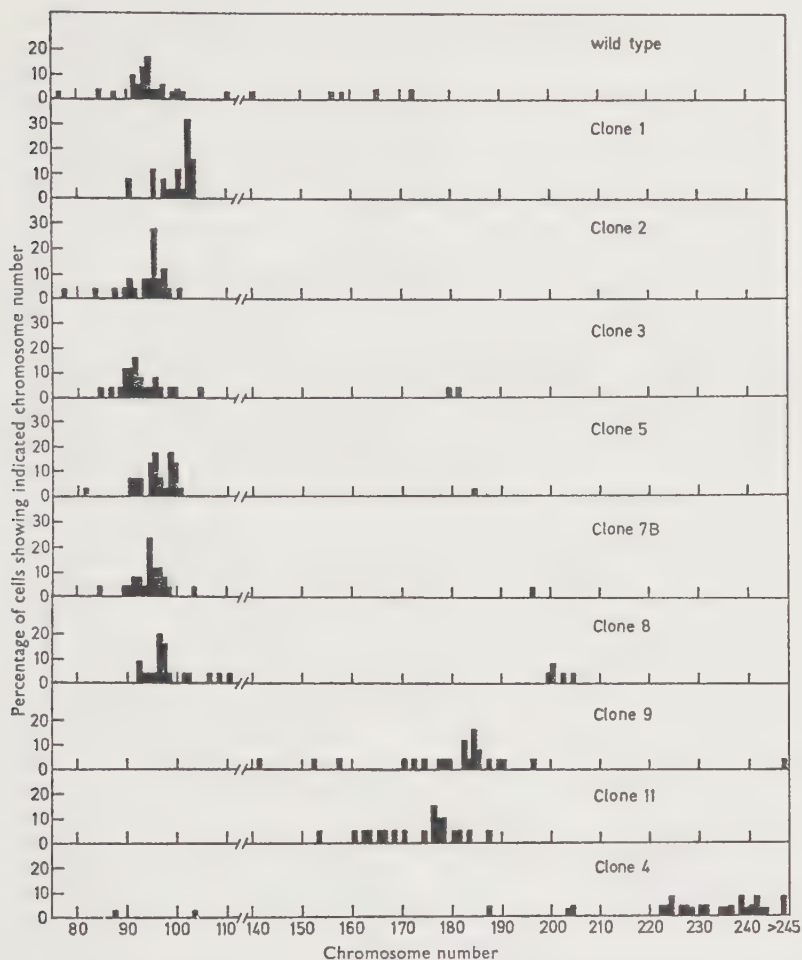


Fig. 1. Distribution of chromosomes in clonal populations of Ehrlich/fibroblast hybrids. The expected chromosome number for a hybrid cell resulting from the fusion of one modal Ehrlich cell and one diploid fibroblast would be 116. Except for grossly polyploid clones, all the hybrid populations have modal chromosome numbers well below that to be expected from the sum of the 2 parental chromosome sets.

(clones 4, 9 and 11), all these hybrids, as soon as they could be analysed, already had between 15 and 25 fewer chromosomes than would be expected in a hybrid containing complete sets of chromosomes from the 2 parent cells; and it has not, in fact, proved possible to obtain a hybrid cell population with an unreduced chromosome complement from this particular combination of parents. This result is very different from that obtained when the Ehrlich, and other tumour, cells were fused with the L cell derivatives, when hybrid cells having a chromosome number approximating to the sum of the 2 parental modes were easily obtained. It thus appears that the chromosomal constitution of the Ehrlich/fibroblast hybrids is much less stable than that of the hybrids between the Ehrlich cells and the L cell derivatives; and it is clear that a large number of chromosomes are eliminated from the Ehrlich/fibroblast hybrids in the early cell divisions. This observation is of some interest for it demonstrates that rapidity of chromosome elimination in hybrid cells can be determined by factors other than differences in the species of origin of the parent cells. Even intraspecific hybrids may undergo drastic reduction in chromosome number when certain combinations of parent cells are used.

The failure to produce any Ehrlich/fibroblast hybrids containing the expected chromosome number for the sum of the 2 parental chromosome sets makes it impossible to draw any conclusion about the dominance or recessiveness of the malignant trait in this combination. Since these hybrids eliminate chromosomes very readily, the chances of selecting malignant segregants from the hybrid cell population will clearly be much greater than in hybrids where the complete chromosome sets of the 2 parent cells tend to be maintained for long periods. This could well explain the high take incidences obtained with Ehrlich/fibroblast hybrids. But the question arises why loss of chromosomes *in vitro* should result in a high incidence of malignant cells in Ehrlich/fibroblast cultures, when loss of chromosomes *in vitro* does not increase the incidence of malignant cells in Ehrlich/A9 cultures. The answer no doubt lies in the nature of the chromosomes lost in the two cases. We have seen that the A9 chromosomes (at least the bi-armed ones) tend to be retained in Ehrlich/A9 hybrids on cultivation *in vitro*. But in the case of hybrids between Ehrlich cells and freshly isolated mouse fibroblasts, which are, unlike A9 cells, poorly adapted to growth *in vitro*, it is quite possible that the fibroblast chromosomes are preferentially eliminated, or, at least, are not preferentially retained. The absence of marker chromosomes other than the T6 translocation in the fibroblasts frustrates direct investigation of this point; but there is now substantial evidence that when cells poorly adapted to growth *in vitro* are fused with others, replication of DNA may lag in the nuclei of the poorly adapted cells and their chromosomes may be preferentially eliminated (Johnson & Harris, 1969; Harris, 1970; Johnson & Rao, 1970; Johnson, Rao & Hughes, 1970; Schwartz, Cook & Harris, 1971). That some fibroblast chromosomes are lost from the Ehrlich/fibroblast hybrids is shown by the absence of at least one of the T6 chromosomes in the great majority of the hybrid cells examined. More decisive observations on the role of chromosome loss in determining the incidence of malignant cells in hybrid cell cultures will be presented in the following paper.

Tumours produced from Ehrlich/fibroblast hybrids

Analysis of the chromosomes of the tumours produced by the injection of these hybrids also revealed the operation of *in vivo* selection, although the results were not as dramatic as those seen with the Ehrlich/A9 hybrids. The results of some typical analyses are shown in Figs. 2-4. In some cases, the modal chromosome number of the

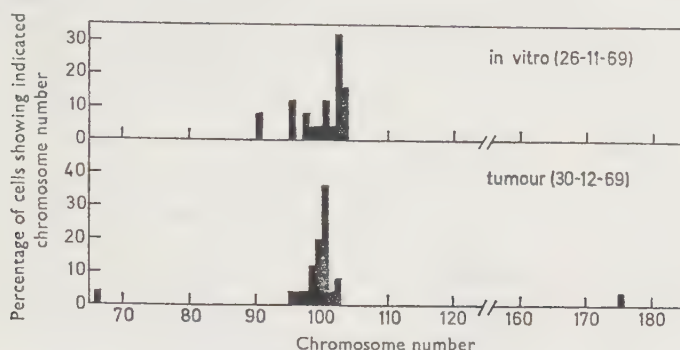


Fig. 2. Distribution of chromosomes in a clonal population of Ehrlich/fibroblast hybrids growing *in vitro* and in a tumour arising from these hybrids. In this case there is only a slight reduction in the modal chromosome number of the cells in the tumour; but the chromosome number of the hybrid cells *in vitro* was already substantially lower than that to be expected from the sum of the 2 parental chromosome sets.

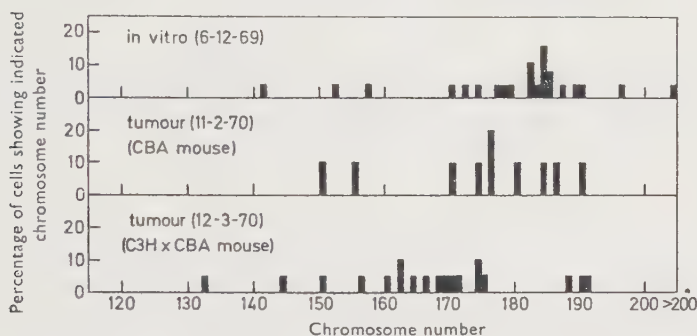


Fig. 3. Distribution of chromosomes in a clonal population of Ehrlich/fibroblast hybrids growing *in vitro* and in tumours arising from these hybrids. The hybrid cells in this case were highly polyploid. The tumours derived from them show a reduction in chromosome number relative to that of the cells injected.

tumour cells was little different from that of the hybrid population injected into the animal (Fig. 2). This finding is not, however, of any special significance, since the hybrid cells had already undergone drastic chromosomal losses *in vitro*. In other cases, the cells in the tumour showed a reduction in modal chromosome number relative to

that of the hybrid cells injected (Fig. 3), thus indicating that selection for cells with reduced chromosome numbers was occurring *in vivo* even in populations of hybrid cells which had already undergone some loss of chromosomes *in vitro*. All tumours showed continued and progressive reduction in chromosome number on repeated transplantation *in vivo* (Fig. 4). These results clearly indicate that the tumours arising in the animal are produced by selection of cells, but some of the hybrid cells already showing a reduced chromosome number *in vitro* can give rise to tumours in which the cells do not show a further reduction in chromosome number.

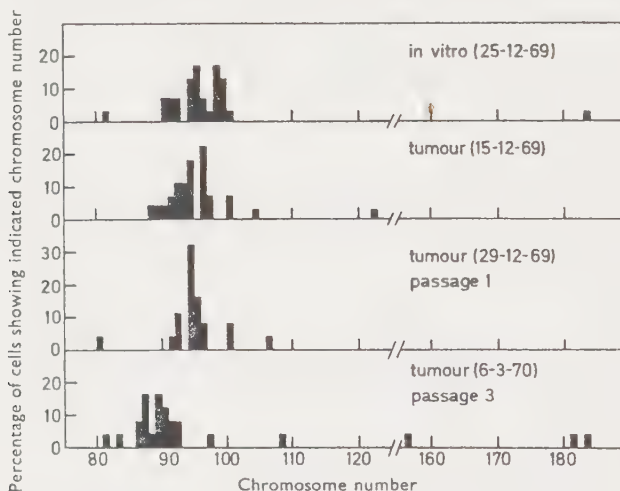


Fig. 4. Progressive loss of chromosomes on continued passage of a tumour arising from a clonal population of Ehrlich/fibroblast hybrids. The initial tumour shows only a slight reduction in modal chromosome number relative to that of the cells injected; subsequent passages show more marked chromosome losses.

DISCUSSION

These observations on Ehrlich/fibroblast hybrids are, as far as the main point of the investigation is concerned, inconclusive. They do not decide whether a diploid fibroblast fused with a malignant tumour cell can suppress malignancy in the hybrid; but they do show how instability of the chromosomal constitution of the hybrid cell may profoundly affect the interpretation of the results in such fusion experiments. In order to decide whether a normal diploid cell can suppress malignancy, it is obviously necessary to construct hybrids that maintain the complete chromosome sets of the two parent cells at least long enough to permit adequate assays to be made. In the following paper we present some observations on such hybrids.

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Table 1. *Growth of Ehrlich/fibroblast hybrids in vivo*

Cell line or clone	All animals		Unirradiated animals		Irradiated animals	
	No. takes (Total no. animals with progressive tumours/total no. inoculated)	Percentage takes	No. takes	Percentage takes	No. takes	Percentage takes
Ehrlich/T6T6 (1)	227/228	99	104/105	98	123/123	100
Ehrlich/T6T6 (2)	170/212	80	85/125	68	85/87	98
Ehrlich/T6T6 (3)	17/18	78	9/11	82	5/7	—
Ehrlich/T6T6 clonal series						
Clone 1	14/14	100	2/2	—	12/12	100
Clone 2	27/28	97	—	—	27/28	97
Clone 3	26/27	96	6/7	—	20/20	100
Clone 4	36/45	80	—	—	36/45	80
Clone 5	25/25	100	14/14	100	11/11	100
Clone 7	18/19	95	11/11	100	7/8	—
Clone 7B	11/12	92	—	—	11/12	92
Clone 8	26/28	93	—	—	26/28	93
Clone 9	10/13	77	—	—	10/13	77
Clone 11	6/10	60	—	—	6/10	60

Table 2. *Chromosomal constitution of Ehrlich/fibroblast hybrids*

Cell line	Date of examination	No. cells examined	Total chromosome no.		No. bi-armed chromosomes	
			Range	Mode	Range	Mode
Ehrlich tumour	13. 9. 69	37	71-94	76	0-2	1
Ehrlich/T6T6 (1)	9. 9. 69	35	83-107	100	0-3	2
	26. 9. 69	25	87-105	99	0-4	2
	27. 10. 69	25	93-104	100	2-3	3
	9. 9. 69	35	83-98	90	1-2	1
Ehrlich/T6T6 (2)	27. 10. 69	25	81-91	88	—	1
	12. 11. 69	30	76-110	94	1-3	1

Table 3. *Chromosomal constitution of clonal populations of Ehrlich/fibroblast hybrids*

Cell line	Date of examination	No. cells examined	Total chromosome no.		Percentage polyploid cells	Chromosome no. of polyploid clones	
			Range	Mode		Range	Mode
Ehrlich T 6 T 6							
(wild type)	12. 11. 69	30	76-110	94	16	—	—
Clone 1	26. 11. 69	25	90-103	102	—	—	—
Clone 2	24. 11. 69	25	77-100	95	—	—	—
Clone 3	26. 11. 69	25	84-104	91	—	—	—
Clone 4	25. 11. 69	27	—	—	> 90	187-251	No mod
Clone 5	25. 11. 69	30	81-100	95-98	—	—	—
Clone 7B	13. 12. 69	25	84-103	94	—	—	—
Clone 8	8. 12. 69	25	95-111	99	—	—	—
Clone 9	6. 12. 69	25	—	—	> 90	141-196	184
Clone 11	6. 12. 69	20	—	—	> 90	153-187	176

THE ANALYSIS OF MALIGNANCY BY CELL FUSION

III. HYBRIDS BETWEEN DIPLOID FIBROBLASTS AND OTHER TUMOUR CELLS

F. WIENER, G. KLEIN

AND H. HARRIS

INTRODUCTION

The Ehrlich tumour used in the present experiments is a subtetraploid line showing a spread of chromosome numbers between 71 and 94. It seemed possible that hybrids with more stable karyotypes might be obtained if the fibroblasts were fused with less aneuploid tumour cells. The SEWA tumour has a mode of 43, and a range of 42-44, chromosomes, and the TA₃ tumour, with a mode of 40 chromosomes, is almost euploid. Both of these tumour cells were therefore fused with normal diploid fibroblasts and a range of clonal populations of the resulting hybrid cells were tested for their ability to grow progressively *in vivo*.

Cell types

The SEWA and TA₃ tumours were taken directly from the mouse peritoneal cavity. The characteristics of the SEWA tumour have already been described (Klein, Bregula, Wiener & Harris, 1971). The TA₃ tumour is an ascitic form of a solid spontaneous mammary adenocarcinoma. The adenocarcinoma originally arose in Dr T. S. Hauschka's laboratory and was converted to the ascitic form in 1951 (Klein, 1951). It has a near diploid chromosome number (Levan, 1956). It carries the H-2^a isoantigen complex derived from the strain A mouse in which it arose, but at a rather low concentration; it readily transgresses H-2 and other histocompatibility barriers. With an inoculum of 10 cells it gives a take incidence of 90–100%, and between 10⁴ and 10⁵ cells intraperitoneally produce an ascites tumour which is lethal in 12–16 days. Fibroblasts were obtained as before by trypsinization of mouse embryos. CBA mice bearing the T6T6 translocations were used to provide the cells for fusion with the SEWA cells; ACA mice provided the cells for fusion with the TA₃ cells. ACA mice were used in this combination because they possessed a unique histocompatibility system. This was desirable for certain studies, not described in the present papers, on the immunological characteristics of these hybrid cells.

Cell fusion

This was carried out as described for the Ehrlich/fibroblast hybrids (Bregula, Klein & Harris, 1971).

Selection of hybrid cells

SEWA/fibroblast hybrids, like Ehrlich/fibroblast hybrids, adhere to the plastic surface of the culture flasks less tenaciously than the fibroblasts themselves, and they may be selected, as described in the preceding paper, by shaking the flasks and transferring the resulting cell suspensions to fresh flasks. Clonal populations of SEWA/fibroblast hybrids were obtained in the same way as the clonal populations of Ehrlich/fibroblast hybrids.

Hybrids between the TA₃ cells and fibroblasts were more difficult to obtain in pure culture. The TA₃ cells grow well as a monolayer on the plastic surface and initially easily outgrow the hybrid cells in the fused cell population. Since no selective procedure was available to separate the hybrid cells from the tumour cells, the only means of obtaining pure cultures of hybrids was to isolate a large number of clones from the fused cell population and screen these. Some 50 primary clones were therefore obtained in the manner described for the Ehrlich/fibroblast hybrids, and those clones that showed a morphology clearly different from that of the tumour cell clones were harvested and grown up as separate subpopulations. Some of these proved on further examination to be composed of hybrid cells.

Assay for tumorigenicity

The SEWA/fibroblast hybrids were injected subcutaneously into syngeneic irradiated newborn mice (*A.SW* × *CBA F*₁ hybrids) and, for comparison, into allogeneic irradiated newborn animals: *A.SW*, *A.SW F*₁ hybrids other than *A.SW* × *CBA*, *CBA* and *CBA F*₁ hybrids other than *A.SW* × *CBA*. The TA₃/ACA hybrids were tested in syngeneic irradiated newborn mice (*A* × *ACA F*₁ hybrids) and also in allogeneic irradiated newborn animals: *A*, *A F*₁ hybrids other than *A* × *ACA*, *ACA* and *ACA F*₁ hybrids other than *A* × *ACA*. Inocula varied from 1.2 × 10⁴ to 3.6 × 10⁶ cells. The hybrid cells were tested for tumorigenicity as soon as large enough populations could be obtained. Subsequent tests were made on cultures grown continuously *in vitro*.

RESULTS

Chromosomal constitution of SEWA/fibroblast hybrids

The hybrid nature of the cells was established by the presence of marker chromosomes from the SEWA parent and the T6 translocation chromosomes from the

fibroblast parent. The hybrid cells also bore both the H-2^s and H-2^k histocompatibility antigen complexes of the two parent cells. Table 1 shows the distribution of chromosome numbers in the wild type population and in 15 clonal populations each derived from a separate primary fusion. The first analyses were made just as soon as adequate numbers of cells could be grown up from the primary clones. The expected modal chromosome number for a hybrid cell produced by the fusion of one fibroblast and one SEWA cell would be 83, with a narrow range of 82–84. It will be seen that, as soon as they could be examined, most of the clones of SEWA/fibroblast hybrids, like the Ehrlich/fibroblast clones, already showed a marked reduction in chromosome number below that to be expected from the fusion of two modal parent cells. However, a few clones did initially show modal chromosome numbers in the region of 80, and *in vivo* tests could be carried out on some of these clones before substantial loss of chromosomes had occurred *in vitro*.

Growth of SEWA/fibroblast hybrids in vivo

Table 2 shows the cumulative take-incidences for those subpopulations that could be adequately tested. It will be seen that in the irradiated syngeneic recipients the wild type population and all clones with reduced chromosome numbers gave take incidences not far short of 100%. But of the 5 clones that initially showed a chromosome complement approximating to the sum of the chromosomes of the two parent cells, 3 (clones 2, 3 and 13) showed a greatly reduced cumulative take incidence. The early tests on these clones showed a very low level of tumorigenicity, comparable to that seen with the SEWA/A9 hybrids; but as loss of chromosomes became apparent on continued passage of the cells *in vitro*, so the take incidence began to show irregularity from one batch of cells to another, and eventually highly tumorigenic subpopulations were obtained from these clones also. This progressive increase in take incidence on continued passage of the cells *in vitro* is illustrated in Table 3. These findings establish that a normal diploid fibroblast can also suppress the malignancy of the tumour cell parent, but it only does so in hybrid cells that retain something approximating to the complete chromosome sets of the two parent cells. The differences in the cumulative take incidences between the SEWA/A9 and the SEWA/fibroblast hybrids are not apparently due to the inferior ability of the fibroblast to suppress malignancy, but to the fact that the chromosomal constitution of the SEWA/fibroblast hybrids is much less stable than that of the SEWA/A9 hybrids.

These results also eliminate the possibility that histoincompatibility plays an important role in determining the observed variation in take incidence. First, marked differences are seen in the take incidences for different clones of hybrids derived from the same two parent cells; and second, these differences are observed even when the clones are tested in irradiated allogeneic animals (Table 2). Other studies have shown that the genetic loci determining malignancy and those determining the expression of histocompatibility antigens segregate independently (Klein, Gars & Harris, 1970).

Table 4 summarizes the chromosomal analyses on some 40 tumours produced from SEWA/fibroblast hybrids in both syngeneic and allogeneic hosts. The results show two striking features. In all cases the range of chromosome numbers seen in the cells of the tumours is very much broader than that to be expected from the fusion of one

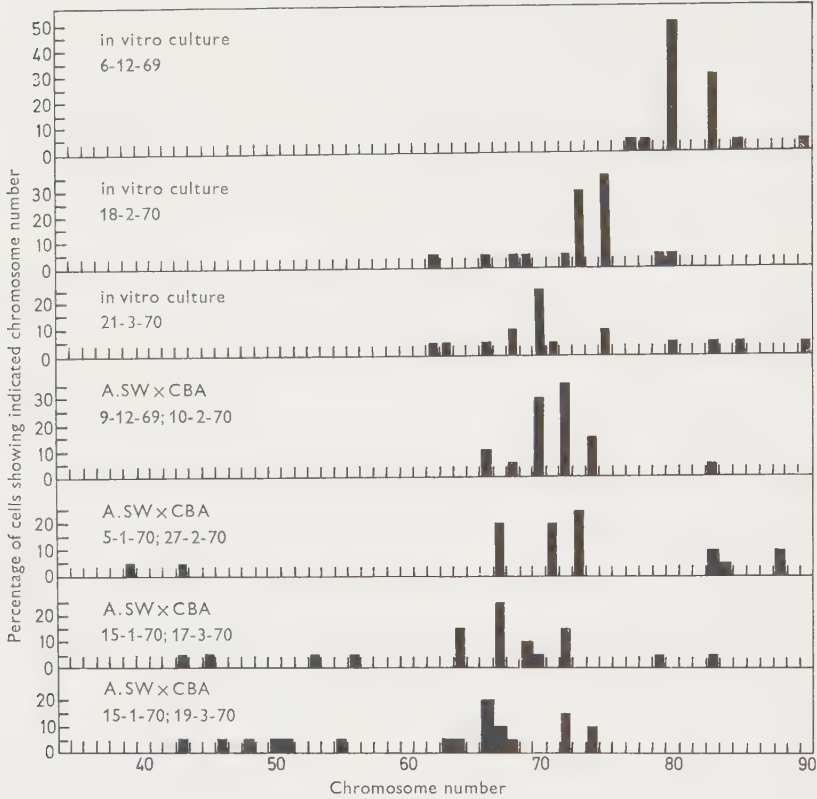


Fig. 1. Chromosomal constitution of SEWA/fibroblast clone 3 and of the tumours produced from this clone. All the tumours show a reduction in chromosome number relative to that of the hybrid cells injected; but the hybrid cells also lose chromosomes on continued cultivation *in vitro*.

SEWA cell and one fibroblast (82-84); and in almost all cases there is a marked reduction in the modal chromosome number relative to that expected from the fusion of one modal SEWA cell and one fibroblast (83). (In the 3 or 4 cases where the modal chromosome number of the cells in the tumour was in the region of 80, this figure was no more than a weak mode in a broad range of chromosome numbers; the tumours contained a high proportion of cells with grossly reduced chromosome numbers and

some cells with chromosome numbers greater than that to be expected from the sum of the parental modes. The development of this type of aneuploidy during the growth of progressive tumours is, of course, well known.) These findings again indicate that the ability of 'complete' hybrid cells, containing unreduced parental chromosome sets, to grow progressively *in vivo* is limited; in all cases where the observations

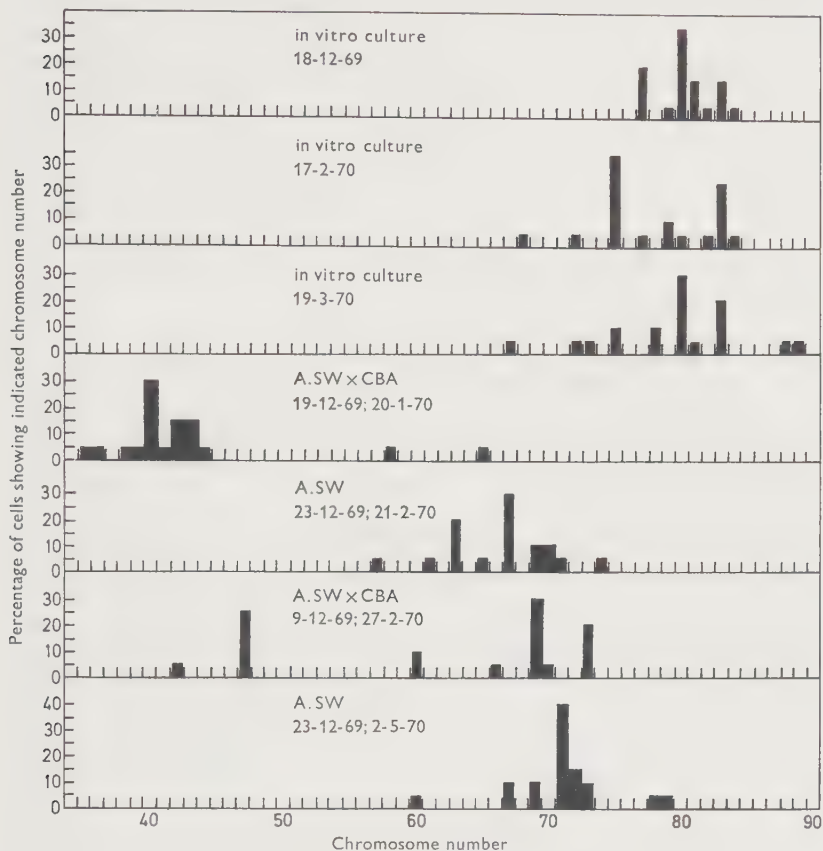


Fig. 2. Chromosomal constitution of SEWA/fibroblast clone 5 and of the tumours produced from this clone. All the tumours show a reduction in chromosome number relative to that of the hybrid cells injected, even though, in this case, the hybrid cells, maintained *in vitro* for the same period, show little or no reduction in chromosome number.

are not obscured by gross aneuploidy, the tumours arising from the injection of the hybrid cell population are not composed of 'complete' hybrids but are dominated by cells from which some chromosomes have been eliminated. This process of selection is illustrated in Figs. 1 and 2, which show the chromosomal constitutions of 2 clonal hybrid populations *in vitro* and those of the tumours produced from these

hybrids. Both clones at the time of injection showed modal chromosome numbers approximating to the sum of the 2 parental modes. The cells in the tumours arising from these hybrids, however, show a reduction in modal chromosome number and occasionally a very drastic reduction. It has been argued (Barski, 1970) that the loss of chromosomes in the cells of the hybrid tumours is of no special significance because the hybrid cells may show comparable chromosome losses *in vitro*. The results shown in Figs. 1 and 2 dispose of this argument. For whereas Fig. 1 shows a reduction in modal chromosome number in both the tumour cells and in the cells maintained *in vitro*, Fig. 2 shows marked reductions in the modal chromosome numbers of the cells in the tumours even when the hybrids maintained *in vitro* for the same period show little or no reduction in chromosome number. Selection for cells with reduced chromosome numbers occurs *in vivo* whether loss of chromosomes occurs *in vitro* or not. The results obtained with the SEWA/fibroblast hybrids are thus fundamentally the same as those obtained with the SEWA/A9 hybrids. In both cases the evidence indicates a very low level of malignancy for hybrid cells in which the complete parental chromosome sets are retained.

TA3/fibroblast hybrids

Since the tumorigenicity of the SEWA/fibroblast hybrids was profoundly modified by chromosome losses *in vitro*, populations of TA3/fibroblast hybrids with very high chromosome numbers were selected for special study. A wild type population and several clonal populations were isolated with modal chromosome numbers substantially higher than the sum of the 2 parental chromosome sets. The modal chromosome numbers of the cells in these populations ranged from 111 to 120, whereas the expected sum of the 2 parental modes was 80. These hybrids had obviously at one stage contained more than one chromosome set from each parent, but at least some of them had undergone substantial chromosomal reduction. In the absence of parental chromosome markers, the hybrid nature of the cells was confirmed by the presence of both parental histocompatibility antigen complexes. The results of the *in vivo* assays on the wild type population and on 2 of the clonal populations are shown in Table 5. It will be seen that the level of tumorigenicity of these hybrids is very low, comparable to that seen with hybrids between tumour cells and A9 cells. The TA3/fibroblast hybrids, like the SEWA/fibroblast hybrids with unreduced chromosome complements, initially produced very few tumours; but the take incidence increased as chromosome loss occurred on continued cultivation of the cells *in vitro*. Analysis of the chromosomes in the tumours produced by the injection of the TA3/fibroblast hybrids again revealed a drastic reduction in modal chromosome number in all cases. Whereas the modal chromosome numbers of the 3 cell populations injected were 117, 114 and 112, no tumour had a mode greater than 103, and the great majority had modes in the region between 80 and 90. The results thus confirm those obtained with the SEWA/fibroblast hybrids. The ACA fibroblasts can suppress the malignant character of the TA3 tumour cell and give rise to an essentially non-malignant hybrid cell population. From this population tumours are produced by selective overgrowth of cells in which some chromosomes have been eliminated.

The general conclusions to be drawn from the present studies, despite the great variety of the experimental material, are clear. The evidence indicates that in all cases where a malignant cell is fused with a non-malignant one, or one of much reduced malignancy, the resulting hybrid cell, provided that it retains the complete chromosome sets of both parent cells, has little capacity for progressive growth *in vivo*. We have not in the present material seen a single tumour in which the chromosomal constitution corresponded to that expected for the sum of the 2 parental chromosome sets. All the tumours arising from the injection of the hybrid cell populations are formed by selective overgrowth of cells from which some chromosomes have been eliminated; and the evidence indicates that the generation of malignant variants in the hybrid cell population requires not merely an overall reduction in chromosome number, but the elimination of some specific chromosome or chromosomes derived from the non-malignant parent cell.

We have used the term suppression of malignancy to describe the non-malignant character of the 'complete' hybrids resulting from the fusion of malignant and non-malignant cells. This term seems to us to be an accurate description of the phenomenon. Whatever heritable character is responsible for the malignancy of the tumour cells, it is not irretrievably lost as a result of cell fusion. All the hybrids we have tested are capable of generating malignant variants when certain chromosomes are eliminated. It is therefore difficult to escape the conclusion that in the hybrid cell the non-malignant partner contributes some factor that suppresses or holds in check the malignant properties of the parental tumour cell: and this suppression is removed when certain chromosomes derived from the non-malignant parent are lost. The frequency with which such malignant segregants are generated in the hybrid cell population appears to be related to the stability of the chromosomal constitution of the hybrid cells. Those hybrids that undergo rapid and extensive chromosome losses *in vitro* give high take incidences when injected into the animal; those that tend to retain complete parental chromosomal sets for longer periods of cultivation *in vitro* give low take incidences.

In Darwinian terms these results can be described very simply. The tumour cells used in the present study (and perhaps all malignant tumour cells) are the end products of selection: these cells have been selected for the ability to grow progressively *in vivo*. When such cells are fused with cells that have not been selected for this property (non-malignant cells), the malignant character of the tumour cells is not expressed in the hybrids. In order to obtain a subpopulation of malignant cells from these non-malignant hybrids, one must select again; and the frequency with which malignant subpopulations can be selected is a function of the degree of genetic variation in the hybrid cell population on which the selection operates. By far the most important source of this variation in the present circumstances appears to be the stability or instability of the chromosome set. In the light of the extensive observations that have been made on the role of cell selection in both experimental and human tumours all this, it seems to us, is hardly surprising.

If our results are to be expressed in Mendelian terms, then one would say that malignancy behaves as if it were a recessive character. In our range of hybrids this is the rule; and we know of no decisive exception in any other studies. But there is no *a priori* reason why malignancy should invariably be recessive, and it may be that on further study of a wider range of material, cases may arise where malignancy behaves as a dominant character. For example, in a case where the non-malignant parent cell is susceptible to a particular oncogenic agent carried by the malignant parent cell, and this agent persists in the hybrid cell, one might expect that the hybrid would be malignant. But the demonstration that malignancy is dominant requires not only that the hybrid cells on injection produce tumours; it must also be shown that the cells in these tumours have the full chromosome sets of the two parent cells. In our material, this has not been found.

We have now to consider what relevance the results of the present investigation have to the genesis of malignant tumours. One theory of the origin of malignancy would fit our findings very well: the idea that malignancy results from a specific genetic loss. If malignancy were the result of a genetic deletion, or of a defect that produced a non-functional gene product, then recessiveness of the malignancy in hybrid cells would be expected, for the non-malignant or normal partner in the hybrid would then be able to complement the defect. If more than one type of genetic loss could produce malignancy, one might expect that hybrids between different kinds of tumour cell might sometimes show complementation and thus be non-malignant. To explore this possibility we are at present examining the behaviour of hybrids produced by fusing together different kinds of malignant tumour cell.

Whatever the precise nature of the genetic lesion responsible for malignancy, the present findings clearly delineate the operation of two processes in the formation of a malignant tumour: the generation of genetic variation in the cell population as a whole and the selection by the body of certain specific variants that are capable of progressive growth. This view of the genesis of malignancy does not require that an oncogenic agent should be highly specific in the genetic lesions that it induces; it would be enough if such an agent raised the level of genetic variation in the exposed cell population to a point where the specific lesion responsible for malignancy, although a random event, had a high probability of occurring. On this view, oncogenic agents such as chemical carcinogens or radiation would not act by converting normal cells to malignant cells, but by generating enough genetic variation to permit malignant variants to occur with predictable frequency. In the hybrid cells that we have examined, the major source of this variation, and hence the major factor influencing the incidence of malignant variants, is chromosomal instability; and the available cytogenetic data do not argue against the operation of a similar source of variation in the malignant tumours of animals and man. But this selectionist interpretation of malignancy implies that the vast majority of the variants produced, and hence the vast majority of the chromosomal aberrations seen in precancerous conditions, are essentially irrelevant to the production of the malignant state. This idea does not, of course, preclude the possibility that in some cases the oncogenic agent, for example a virus, might interact with the cell in a more specific way.

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Table 1. Chromosomal constitution of SEWA/fibroblast hybrids

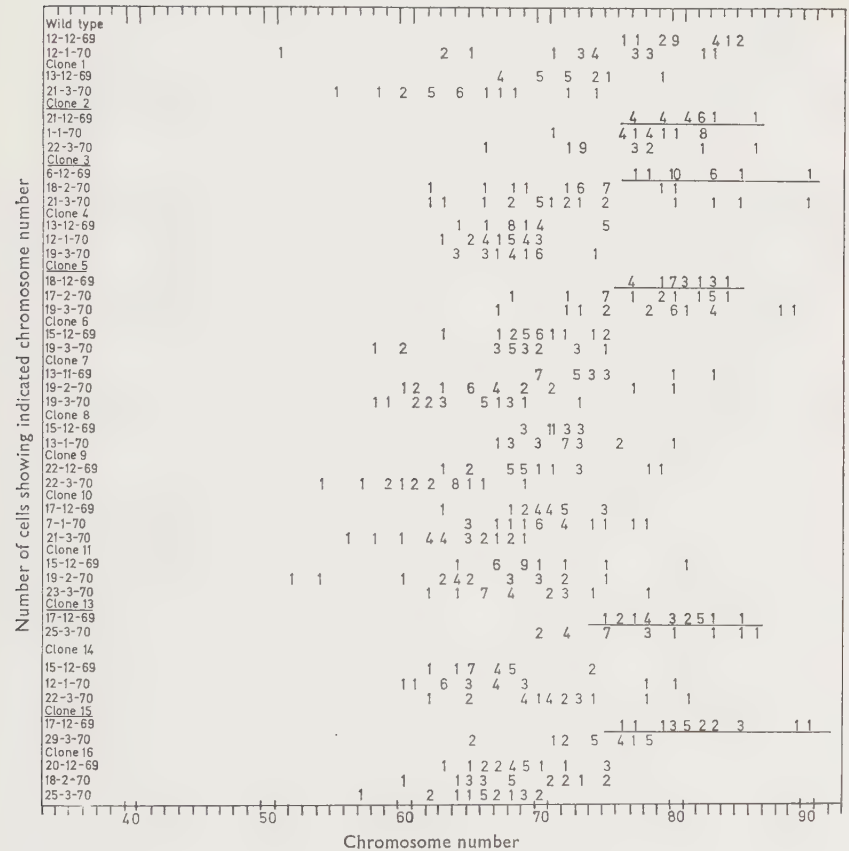


Table 2. *Growth of SEWA/fibroblast hybrids in vivo*

Cell line	Syngeneic recipients <i>A.SW</i> and <i>A.SW</i> F_1 <i>CBA</i> and <i>CBA</i> F_1 (<i>A.SW</i> $\times$ <i>CBA</i> F_1 hybrids other than <i>A.SW</i> $\times$ <i>CBA</i> hybrids other than <i>A.SW</i> $\times$ <i>CBA</i>)					
	Percent- tage takes		Percent- tage takes		Percent- tage takes	
	No. takes		No. takes		No. takes	
SEWA/T6T6 wild type	15/16	94	5/15	33	—	—
SEWA/T6T6						
Clone 1	4/4	(100)	8/16	50	1/4	25
Clone 2	11/19	58	1/8	13	2/11	18
Clone 3	13/27	48	1/40	3	2/14	14
Clone 4	7/7	100	0/9	0	1/5	20
Clone 5	6/6	100	6/19	32	13/16	81
Clone 6	3/3	(100)	5/5	(100)	10/10	100
Clone 7	7/7	100	14/21	67	2/2	(100)
Clone 10	12/12	100	9/9	100	4/6	67
Clone 13	16/31	51	0/17	0	1/27	4
Clone 14	15/15	100	7/24	29	19/20	95
Clone 15	3/3	(100)	11/23	48	13/13	100
Clone 16	18/20	90	5/10	50	7/10	70

All tests were made in irradiated newborn mice.

Table 3. *Progressive increase in take incidence of SEWA/fibroblast hybrids on continued passage in vitro*

Clone 2		
Date of inoculation	No. cells injected	No. takes
5. 1. 70	3.2×10^4	0/4
13. 2. 70	2.4×10^5	0/3
27. 2. 70	1.2×10^5	3/4
20. 3. 70	8.8×10^5	3/3
7. 4. 70	7.8×10^5	3/3
21. 4. 70	4.3×10^5	2/2

Table 4. *Chromosomal constitution of tumours produced from SEWA/fibroblast hybrids*

Clone	No. of chromosomes		Date of inoculation	Date of examination	Genotype of test animal	
	Mode	Range				
1	72	58-75	18. 12. 69	15. 1. 70	A. SW × CBA	S
2	63	63-83	21. 4. 70	11. 6. 70	CBA × C ₃ H	A
	80	69-85	14. 4. 70	25. 6. 70	CBA	A
3	72	66-83	9. 12. 69	10. 2. 70	A. SW × CBA	S
	73	39-88	5. 1. 70	27. 2. 70	A. SW × CBA	S
	67	43-83	15. 1. 70	17. 3. 70	A. SW × CBA	S
	* { 81-83	81-90	15. 1. 70	19. 3. 70	A. SW × CBA	S
	{ 66	43-74				
4	85	58-90	15. 1. 70	17. 3. 70	A. SW × CBA	S
	62	58-76	19. 12. 69	20. 1. 70	A. SW × CBA	S
	57	57-83	29. 12. 69	14. 2. 70	A. SW × CBA	S
	65	40-84	13. 2. 70	5. 3. 70	A. SW	A
	69	63-83	5. 3. 70	20. 3. 70	A. SW	A
	60	44-82	20. 5. 70	4. 6. 70	A. SW	A
5	41	36-65	19. 12. 69	20. 1. 70	A. SW × CBA	S
	70	60-79	23. 12. 69	2. 5. 70	A. SW	A
	69	42-73	9. 12. 69	27. 2. 70	A. SW × CBA	S
	67	57-82	23. 12. 69	21. 2. 70	A. SW	A
6	75	48-75	22. 1. 70	5. 3. 70	CBA	A
	66	53-68	22. 1. 70	5. 3. 70	CBA (ascites tumour)	A
7	52	41-73	3. 2. 70	26. 3. 70	CBA	A
	67	39-73	23. 12. 69	4. 2. 70	A. SW	A
8	65	45-70	29. 12. 69	3. 2. 70	A. SW × CBA	S
10	68	50-83	30. 12. 69	4. 2. 70	A. SW × CBA	S
	65	48-83	2. 2. 70	22. 3. 70	A. SW × C ₃ H	A
11	67	41-90	3. 2. 70	24. 3. 70	A. SW × CBA	S
13	62	46-71	15. 5. 70	8. 7. 70	A. SW × CBA	S
14	66	39-75	3. 2. 70	24. 3. 70	A. SW × C ₃ H	A
	69	66-73	7. 4. 70	25. 6. 70	A. SW × CBA	S
	78	44-82	4. 6. 70	28. 6. 70	A. SW × CBA	S
	76	40-81	4. 6. 70	28. 6. 70	A. SW × CBA	S
	78	64-83	18. 6. 70	9. 7. 70	A. SW × CBA	S
16	75	70-80	25. 6. 70	10. 7. 70	CBA	A
	60-67	40-72	12. 1. 70	27. 2. 70	A. SW × CBA	S
	60	48-60	13. 1. 70	5. 3. 70	A. SW × CBA	S
	57-68	50-83	26. 1. 70	7. 3. 70	A. SW × CBA	S
	65	48-83	16. 3. 70	28. 6. 70	A. SW × CBA	S
Wild type	71	54-81	24. 11. 69	13. 1. 70	A. SW × CBA	S
	68	52-74	15. 1. 70	21. 2. 70	A. SW × CBA	S

S = syngeneic host; A = allogeneic host.

* The tumour appeared to contain 2 cell populations.

Table 5. *Growth of TA₃/fibroblast hybrids in vivo**

Cell line	Chromosome number		Syngeneic recipients (<i>A</i> × <i>ACA</i> <i>F</i> ₁ hybrids)		<i>A</i> strain mice		<i>ACA</i> strain mice	
	Mode	Range	No. takes	Percentage takes	No. takes	Percentage takes	No. takes	Percentage takes
Wild type	117	100-117	19/51	37	14/68	21	2/42	5
Clone 5	112	110-121	2/15	13	0/7	0	0/11	0
Clone 7	114	66-121	0/6	0	4/23	17	12/48	25

* All tests were made in irradiated newborn mice.

Susceptibility of Primate-mouse Hybrid Cells to SV40¹

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The recognition of hybrid cells in culture currently rests on several criteria: morphologic, karyotypic, enzymologic, immunologic (cell surface antigens) and survival after treatment with techniques that eliminate the parental cells. In hope of adding another parameter to this list, we investigated the susceptibility of primate-mouse hybrid cells to infection by a virus which is limited in its host range — simian virus 40 (SV40) which replicates only in primate cells (Sweet and Hilleman, '60).

Hybrid cells were prepared between cells susceptible to lytic infection by SV40 (human and African green monkey) and cells resistant to SV40 (mouse). In addition, SV40-transformed cells were used as parents for hybridization. The resultant hybrids were characterized by as many parameters as possible and challenged with SV40 DNA. Since some transformed cells have been shown to be resistant to superinfection with whole virus but not with the viral DNA, presumably due to a surface phenomenon (Swetley et al., '69; Barbanti-Brodano et al., '70), these cells were challenged with SV40 nucleic acid and evaluated for their ability to form whole infectious virus particles. Differences in susceptibility to SV40 between various human cell strains have also been reported but infection with SV40 DNA equalizes these differences in susceptibility (Aaronson, '70).

MATERIALS AND METHODS

Cell cultures. The cell cultures used are described in table 1. All cells were grown in Eagle's basal medium in Earle's balanced salt solution (AutoPow, Flow Laboratories) supplemented with 10% fetal calf serum, 5-bromo 2'deoxyuridine (5-BUDR, 30 μ g/ml), 8-azaguanine (8-AZG, 3 μ g/ml), and HAT (3×10^{-6} M glycine, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-3} M thymidine) (Littlefield, '64) were added to some of the media as indicated in table 1.

Cell fusion. The technique of cell fusion was essentially that described previously (Knowles et al., '68) using Sendai virus inactivated by β -propiolactone. Selection of hybrid cell colonies was facilitated by utilization of parental cells with enzyme deficiencies and by use of lymphoid cells that do not attach to the glass.

Karyology. Chromosomes were examined by the technique of Moorhead and Nowell ('64) from cell cultures at the same passage level as those tested for SV40 formation after infection with SV40 DNA.

SV40 DNA infection. Cells were infected with SV40 DNA in the presence of DEAE Dextran as previously described (Swetley et al., '69). After a 72-hours in-

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TABLE 1
Derivation of hybrids and their characteristics

Hybrid	Parental line	Characteristics	SV40 DNA replication	Chromosomes			Cell surface antigens	Isozymes	SV40 t-antigen
				Total number	Bi-armed	Telo-centric			
IP ¹		Growth on glass in HAT	—	66	29	37			
2P		Growth on glass in HAT	—	81	32	49		Primate * LDH G6PD PEP C MOR ^{1,2} Cathodal esterase	
3P		Growth on glass in HAT	—	80	23	67			
5P		Growth on glass in HAT	—	73	28	45			
	WBC	Monkey peripheral white blood cells	ND	60	38	22			
	IR	C3H mouse 8-AZG-resistant	—	57	18	39			
4W10 ³		Growth on glass in HAT	—	61	28	33	Human Mouse H-2 *		
4W7		Growth on glass in HAT	—	63	24	39	Human Mouse H-2 *		
3W4		Growth on glass in HAT	—	62	24	38	Human Mouse H-2 *		
1W1		Growth on glass in HAT	—	57	20	37	Human Mouse H-2 *		
	WBC	Human peripheral white blood cells	ND	46	35-36	10	Human		
	IR	No growth on glass C3H mouse 8-AZG-resistant	—	57	18	39	Mouse H-2 *		
HM ₂ P ₃		Growth in HAT	—	53	12	41	Human Mouse H-2 *		
	WI-38	Human fibroblasts	+	46	36	10	Human		
	cl-1D	C3H mouse BUDR resistant	—	52	9	43	Mouse H-2 *		
FAM-1-TK ⁺		Growth in HAT	—	60	11	49	Human Mouse H-2 *		
TK ⁻		Growth in HAT	—	54	10	45	Human Mouse H-2 *		

			46	35	11	ND	
	Human fibroblast from biopsy	+					
	C3H mouse BUdR resistant	—	52	9	43	Mouse H-2 *	
WR-1 *	Growth in HAT	—	140-163	20-38		Human Mouse H-2 *	
WR-5	Growth in HAT	—	63-83	4-12		Human Mouse H-2 *	
	Human diploid fibroblasts	+	46	36	10	Human	
	BALB/c mouse 8-AZG-resistant	—	64-76	3-5		Mouse H-2 *	
HMSV *	Growth in HAT	—	51	8	43	Human Mouse H-2 *	—
	Human fibroblast SV40 transformed	ND	80	ND	ND	ND	+
	C3H mouse BUdR resistant	—	52	9	43	Mouse H-2 *	—
VT-7 *	Growth in HAT	+	76	4	72	Human Mouse	+
	SV40-transformed human 8-AZG-resistant	+	74	64	10	Human	+
	Normal mouse embryo fibroblast	—	40	0	40	Mouse	—
	Growth in HAT	—	57	14	43	Human Mouse H-2 *	
102-2 cl 3	Growth in HAT	—	60	17	42	Human Mouse H-2 *	
102-2 cl 4	Growth in HAT	—	88	21	66	Human Mouse H-2 *	
102-2 cl 7	Growth in HAT	—	55	10	43	Human Mouse H-2 *	Human Cathodal esterase
133-5	Growth in HAT	—	46	35	11	Human	
	Human Lesch-Nyhan Syndrome fibroblast	+	52	9	43	Mouse H-2 *	
	C3H mouse BUdR resistant	—	115	12	103	Human Mouse H-2 *	
193-1 cl 1	Growth on glass in HAT	—	68	3	65	Mouse H-2 *	Primate * PGM GPI ADA Pep B
	BALB/c mouse BUdR resistant	—					
EB-2 *	Human lymphoid cell line	+	46	36	10	Human	—

TABLE 1 (continued)
Derivation of hybrids and their characteristics

Hybrid	Parental line	Characteristics	SV40 DNA replication	Chromosomes			Cell surface antigens	Isozymes	SV40 t-antigen
				Total number	Bi-armed	Telo-centric			
229-2 cl E		Growth on glass in HAT	+	101	91	10	Human		+
	W18-2	Human lymphoid cell line from "normal" patient (hereditary spherocytosis)	+	46	35	11			-
	W18V _{A2} (w) ⁴	SV40-transformed human 8-AZG-resistant	+	74	64	10			+
243-3 cl A		Growth on glass in HAT	-	109	5	104	Human Mouse		
	WBC	Human peripheral white blood cells	ND	46	35	11	Human		
	IT ¹	No growth on glass							
250-1 cl 1 cl 5		Swiss mouse BUDR-resistant	-	65	0	65	Mouse		
		Growth in HAT	+	136	4	132			+
		Growth in HAT	+	135	6	129			+
	W18V _{A2} (w) ⁴	SV40-transformed human 8-AZG-resistant	+	74	64	10			+
	IT ¹	Swiss mouse BUDR-resistant	-	65	0	65			-

+ or - indicates presence or absence of SV40-T-antigen, cl, clone.
¹ Panel of 13 tested; those listed showed primate-mouse patterns, all others showed only mouse patterns.
² Obtained from Dr. V. Miggiano.
³ Obtained from Dr. Howard Green.
⁴ Obtained from Dr. Howard Green.
⁵ Obtained from Dr. Mary Weiss.
⁶ Obtained from Dr. J. Dancis.
⁷ Obtained from Dr. Del Dubbs.
⁸ Obtained from Dr. W. Henle.
⁹ Obtained from Dr. F. Ruddle.

cubation, the cells were scraped into their medium, disrupted by sonic oscillation, and the samples then treated for 30 minutes at 37°C with 20 µg/ml deoxyribonuclease I (Worthington) in order to eliminate the remaining input SV40 DNA. Complete, newly synthesized, virus particles were assayed by undiluted passage of this preparation into confluent tube cultures of CV-1 cells which were then monitored for SV40 cytopathology (CPE). Each experiment was controlled by the inclusion of SV40 DNA infected CV-1 and mouse cells. Samples of the DNase treated cell extract from infected CV-1 cells yielded whole virus on passage, indicating the integrity of the input DNA, whereas DNase-treated samples of infected mouse cells did not produce SV40 CPE on passage, indicating that any possible residual infectivity of the SV40 DNA was completely destroyed.

SV40 T-antigen immunofluorescence. SV40 T-antigen immunofluorescence was visualized by the indirect technique (Pope and Rowe, '64). Acetone-fixed coverslips were first incubated with antiserum obtained from SV40 tumor-bearing hamsters (Flow Laboratories) and then with fluorescein-conjugated rabbit anti-hamster gamma globulin.

Isozymes. Starch gel electrophoresis was performed on homogenates of the monkey-mouse hybrid cells by Dr. V. M. Chapman in Dr. Frank Ruddle's laboratory (for references to technique, see Ruddle et al., '70). The enzymes investigated include isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PD), phosphoglucomutase (PGM), lactic dehydrogenase (LDH), peptidase A (Pep A), peptidase B (Pep B), peptidase C (Pep C), phosphoglucoisomerase (PGI), deaminase (ADA), malate: NAD oxidoreductase (MOR^{1&2}), glutamic-oxaloacetic acid transaminase (GOT), NADP-malate dehydrogenase (MOD) and indophenol oxidase (IPO).

The human cathodal esterase was assayed as previously reported (Bartholomew et al., '70).

Cell surface antigens. Cell surface antigens were determined by mixed agglutination as described previously (Kano et al., '69).

RESULTS

At the time the AGMK-mouse hybrid cells were challenged with SV40 DNA, at least one-fifth and possibly two-fifths of the AGMK genome was represented in clone 2P (see table 1). These calculations are based on either the mean number of bi-armed chromosomes in excess of the number present in the IR mouse parent (14) or on the number in excess of the mean value for the IR parent (24). In addition, four of the panel of 13 isozymes tested, namely, LDH, G6PD, MOR^{1&2} and Pep C, appear to be of both primate and mouse origin. The human cathodal esterase present in monkey kidney cells, was found in the 2P hybrids as well. Even so, these cells did not support production of whole virus on challenge with SV40 DNA. Clones 3P and 5P contained fewer primate chromosomes, and of the 13 isozymes tested, only Pep C and LDH were present in colony 5P, these cells also did not support SV40 replication.

Because of the availability of hybrids or of human parental cell lines against which selection pressures could be exercised, many more human-mouse hybrids were tested. The well-characterized W series (see table 1) contained between two and ten human chromosomes, in addition to the mouse complement of chromosomes, both human and mouse surface antigens and a G6PD isozyme pattern characteristic for hybrids. These hybrids did not produce SV40 upon challenge with SV40 DNA. Since they contained both G6PD and hypoxanthine guanine phosphoribosyltransferase (HGPRT) mapped in human cells to the X-chromosome (Migeon et al., '68), an association between the X-chromosome and susceptibility to SV40 is unlikely. The hybrid cells of the FAM-1-TK series are also incapable of replicating SV40 DNA to form whole virus. These cells contain very few human chromosomes. (2 in TK⁺ and 1 in TK⁻) but are derived in such a manner that the thymidine kinase genes from the human parental cell are required for growth in HAT. Consequently, the possibility of the human Group E "thymidine kinase chromosome" (Matsuya et al., '68; Migeon and Miller, '68) contributing an essential factor necessary for SV40 production is also eliminated. These hybrids

are derived from cells of a patient with Fanconi's anemia, which are known to be highly susceptible to SV40 (Todaro et al., '66). The HM₂P₂ hybrid also contains the thymidine kinase chromosome from human cells, but this chromosome is derived from the normal (WI-38) human cell strain. These cells also do not produce infectious virus after challenge with SV40 DNA. Expanding the number of human chromosomes in the hybrid by use of Ruddle's WR-1 line with a mean of 22 human chromosomes, representing groups A, B, C, E and F and possibly the telocentric groups D and G, also from WI-38, does not change the nonreceptive state of the hybrids to SV40 DNA. It is no surprise that WR-5, a product of the same hybridization, which has lost considerably more human chromosomes (remaining $\bar{x} = 4$), is also insusceptible to SV40 DNA (for details of karyology, see Klebe et al., '70).

Two hybrids derived from SV40-transformed human parental cells were then challenged with SV40 DNA. HMSV cells no longer contained the SV40 T-antigen at the time of challenge, and human chromosomes could no longer be identified, although the cells were growing in HAT hence contained the human thymidine kinase gene and also showed human surface antigen. This hybrid was incapable of processing the input SV40 DNA into whole virus. The VT-7 hybrid, still SV40 T-antigen-positive at the time of challenge, was capable of SV40 DNA replication. This line also contained 72 telocentric chromosomes, as compared to 40 in the mouse parent and ten in the human parent, suggesting that a telocentric chromosome of human origin, rather than a biamed chromosome, could be essential for the replication of SV40 DNA. On a morphological basis, human chromosome groups A, E, and F are represented in this hybrid. Sendai virus-induced cell fusion of 5×10^6 VT-7 cells with 5×10^6 AGMK cells did not yield SV40 virus. This result is not surprising, since, using the same technique, whole virus could not be rescued from the W18Va₂ (w) parent.

Human Lesch-Nyhan syndrome fibroblasts fused with mouse cl-1D fibroblasts gave rise to a series of hybrid colonies (102-2), containing human chromosomes, as judged on a morphological basis, and

exhibiting human species and transplantation antigens on the cell surface (for details, see Kano et al., '69). Each of these hybrids contains one human group E chromosome, or at least the thymidine kinase gene from the human cell, translocated to another chromosome. In addition, each of these colonies contains a small definite number of human chromosomes, possibly groups C, D and F (for karyotypes, see Kano et al., '69). These colonies and the 133-5 hybrid behave negatively when challenged with SV40 DNA.

A hybrid clone (193-1) derived by fusion of an SV40-transformed mouse parent (MKS-Bu-100) and a human lymphoid cell line (EB-2) derived from a Burkitt's lymphoma, did not produce SV40 on challenge with SV40 DNA. Sendai virus induced cell fusion of 193-1 cells with an equivalent number of AGMK cells resulted in the rescue of SV40 virus. These results are comparable with those obtained with the MKS-Bu-100 parent (Kit, '70) from which virus could easily be rescued by cocultivation or fusion with AGMK cells. This indicates that the whole viral genome is present in the hybrid cells, but is not replicated, presumably because of a cellular deficiency. Challenge of EB-2 with SV40 DNA resulted in viral replication.

The intraspecific hybrid 229-2E was derived using an SV40-transformed human parent, W18Va₂ (w), also the parent of VT-7, and cells from a normal human lymphoid cell line. Challenge of the hybrid cells with SV40 DNA produced virus, as expected.

Fusion of human peripheral white blood cells with BUdR-resistant Swiss mouse fibroblasts yielded a hybrid 243-3 colony A. The human genome is represented by at least five biamed chromosomes, and human species antigen is detectable. These hybrid cells are incapable of SV40 production on challenge with the viral DNA.

The 250-1 hybrid colonies were obtained from fusion of SV40-transformed W18Va₂ (w) human line with a fibroblastic mouse parent (1T). These hybrids were T-antigen positive and on challenge with SV40 DNA replicated the SV40 genome and made virus.

DISCUSSION

From the results presented in table 1, it appears that only those hybrids derived from the same SV40-transformed primate parent (W18Va₂ (w)) were capable of supporting SV40 replication. The W18Va₂ (w) line contains defective SV40 genomes as fusion of these cells with AGMK does not give rise to infectious virus. All of the other hybrids behaved as their nonpermissive mouse parent, in their failure to produce SV40 after challenge with SV40 DNA. HMSV, derived from an SV40-transformed human parent and a normal mouse parent, did not make virus from the input SV40 DNA. This hybrid no longer contained SV40 T-antigen, however, indicating that the human chromosome(s) necessary for the maintenance of the viral genome may be the one(s) necessary for viral replication.

Although 193-1 originated from an SV40-transformed mouse cell and a human cell, challenge with SV40 DNA did not result in virus production. Since these cells already contain intact SV40 genome, as demonstrated by rescue of SV40 after fusion with AGMK, the long period of cultivation between the initial hybridization and the SV40 challenge must have resulted in selection of cells unable to replicate virus. This selection, possibly chromosome loss, is probably an immediate event, with cell destruction of most of the original human-mouse hybrids by SV40. Only a variant hybrid, defective in its ability to replicate the virus, would survive.

A more difficult problem is the inability of any hybrid derived from untransformed cells to replicate the viral DNA. All mouse-primate hybrids lose a considerable proportion of the primate genome soon after hybridization (Weiss and Green, '67). Because of the number of different parental cells fused to make hybrids, the different selection schemes used, and the production of these hybrids in different laboratories, it is necessary to postulate that these cells preferentially lost that portion of the primate genome necessary for SV40 replication. These results are in some ways analogous to those of Basilico et al. ('70) who challenged mouse-hamster hybrid cells with polyoma virus. These hybrids tended to lose mouse chromosomes in culture. Only

those hybrids that contained at least a diploid number of mouse chromosomes were capable of supporting polyoma virus multiplication. Hybrids containing a markedly reduced complement of mouse chromosomes could not support viral replication.

Presuming, then, that all of the primate chromosomes incorporated into a hybrid cell are capable of expressing the functions necessary for SV40 production, either a large portion of the primate genome is necessary before SV40 is made, or those chromosomes necessary for SV40 production are among the first lost in an interspecific hybrid. SV40 thus joins polyoma virus (Basilico et al., '70) and polio (Kusano et al., '70) in that the requirements needed for its reproduction are not found in every hybrid cell.

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SURFACE ANTIGENS AND RELEASE OF VIRUS IN HYBRID CELLS PRODUCED BY THE FUSION OF A9 FIBROBLASTS WITH MOLONEY LYMPHOMA CELLS

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Murine leukemia viruses infect their target cells permissively [16, 7, 19]: the proliferation of the leukemia cells is accompanied by the production of mature virus particles [7]. The infected cells also express new, virus-induced surface antigens [1]. The exact relationship between the surface antigens and the virus particles is not known. In a comparative study of a number of Moloney virus-induced leukemias, no correlation was found between the amount of virus released and the concentration of virus-specific surface antigens [10, 11]. There is usually a correlation between the concentration of such antigens and immunosensitivity.

We have recently isolated two Moloney lymphoma lines from the same original tumour, differing in immunosensitivity [4] and in the amount of virus released [5, 6].

In the present study, these two lymphoma sublines were fused with A9 fibroblasts and the resulting hybrid lines were compared with respect to antigenic characteristics and virus release. The interaction was interesting and unexpected. The suppression of the virus-induced membrane antigen seen in the immunoresistant subline was abolished by fusion with the A9 cell. On the other hand, the lymphoma cell suppressed the release of C-type virus particles by the A9 cell. These results lend further support to the view that the quantity of membrane antigen and the release of infectious virus are not coupled.

MATERIALS AND METHODS

Cells

The A9 mouse fibroblast line was derived by Littlefield [15] from the L cell line by selection for 8-

azaguanine-resistance. The cells lack the enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRT). This defect prevents growth in the selective HAT medium [20]. Since the L strain was derived from a C3H mouse, the A9 subline carries the H-2^k isoantigen complex. It also expresses two other surface antigens: a virus-induced antigen, which is also present on cells infected by the Friend, Moloney, Rauscher or Graffi virus (the FMRG1 antigen), and the L antigen, which is present on the L cell strain and its derivatives [14]. This antigen was assumed to be determined by the C-type particles (the so called "L cell virions"), carried by L cells [14]. Although indistinguishable morphologically from the murine leukemia viruses, the L cell virion has no demonstrable leukemogenic activity [9]. We have recently found [6] that exposure of sensitive indicator cells (JLS-V9) to the medium from cultures of L cells results in the induction of the Moloney-type membrane antigen in the indicator cells.

The origins of the YAC lymphoma and its immunoresistant subline (YACIR) have been described previously [4, 5]. The tumours were maintained in the ascites form by weekly i.p. inoculations of 10⁶ cells into A/Sn mice. The 230th transfer generation of YAC and the 90th subline passage of YACIR were used for the fusion experiments.

Cell fusion and selection of hybrid lines

The two cell types were fused together by the inactivated Sendai virus technique as described by Harris & Watkins [8]. YAC and YACIR cells, like most lymphoid cells, fuse poorly: a relatively high input of these cells and high doses of inactivated virus are required to produce reasonable numbers of heterokaryons. Satisfactory results were obtained with 10⁷ YAC or YACIR cells, 2 × 10⁶ A9 cells and 2 000 hemagglutinating units of inactivated virus. The lymphoma cells do not adhere to glass and may be removed from the fused cell cultures simply by two or three changes of medium. A9 cells do not grow in HAT medium, so that maintenance of the fused cell cultures in this medium permitted the growth of hybrid cells only. The hybrid clones appeared in the cultures about 3 weeks after cell fusion.

Chromosome preparations

Chromosome preparations of the ascites tumour cells and of the cells grown *in vitro* were made by the air drying technique of Rothfels & Siminovich [18]. The cells were exposed to Colcemid (0.1 µg/ml) for 2 h, treated with sodium citrate solution (0.9%) and fixed with acetic acid-methanol (1:3). They were then spread on wet slides, air-dried and stained with buffered Giemsa solution. 25 metaphases were examined in each sample.

Antisera

Isoantisera were produced by immunizing groups of 10–13 adult mice with pooled cell suspensions, prepared from spleen, kidney and liver, injected

subcutaneously once every 2 weeks for 14–24 weeks. The mice were bled 7–10 days after the last injection. C57Bl DBA/2 anti C3H serum was used to demonstrate H-2^k antigens. This serum had no detectable anti-Moloney activity, since it failed to react with Moloney virus-infected JLS-V9 cells (BALB/C origin, H-2^d). C57Bl × C3H anti DBA/2 serum was used to demonstrate H-2^d antigens. This serum failed to react with the Moloney virus-induced YBA lymphoma (CBA origin, H-2^k), and was thus free of Moloney-specific antibodies. The two H-2 antisera titrated to 1:200 and 1:64 respectively in our standard cytotoxic test against YAC (H-2^d) target cells.

Anti-Moloney serum was collected from (ABY × DBA/2) F₁ hybrid mice, 90–150 days after immunization with a 10% homogenate of a syngeneic Moloney lymphoma (YDYA). Three inoculations were given at 1 week intervals. This serum titrated to 1:32 in the standard cytotoxic test using YAC target cells.

A typing serum for the L antigen was collected from (C3H × DBA/2) F₁ hybrid mice immunized with A9 cells subcutaneously at fortnightly intervals for 21 weeks. The mice were bled 7–10 days after each inoculation, beginning with the third immunization, and the sera were pooled. The serum titred to 1:3 000 in indirect membrane immunofluorescence tests against A9 target cells. It did not react with Moloney virus-infected JLS-V9 cells or with YAC lymphoma cells. All sera were stored frozen at –20°C.

Cytotoxic tests were performed as previously described [11].

Indirect membrane immunofluorescence tests were performed on viable cells as previously described [13]. Suspensions containing at least 80% living cells were used. Cells were washed twice by centrifugation in Hanks' balanced saline solution containing 1% gelatin. Mouse sera were serially diluted in this solution. All sera were tested undiluted and at dilutions of 3, 10, 30, 100, 300, 1 000 and 3 000. Fluorescein-labeled goat anti-mouse gamma globulin, obtained from Hyland Laboratories (Los Angeles, Calif.), was used in a 1:20 dilution. Approximately 100 cells were classified in each sample, according to the following criteria. All cells showing bright green granular, sectorial or ring fluorescence were counted as positive. Cells showing faint grey autofluorescence, but not brilliant green membrane staining, were counted as negative. Dead cells showing diffuse cytoplasmic fluorescence were not counted. For each sample, a fluorescence index was calculated by subtracting the percentage of negative cells from the corresponding value in a sample of cells exposed to a control serum and dividing the difference by the latter figure. As a rule, 80–90% of the cells were negative in control samples.

Mixed hemadsorption test

Cells were seeded in the wells of Falcon microtest plates (Falcon Plastics, Los Angeles, Calif.) and incubated for 18–40 h at 37°C in 5% CO₂. A micro-modification of the mixed hemadsorption technique [3] was used [21]. Veronal-buffered saline containing

5% gelatin was used as the diluent and washing fluid. The indicator cells were prepared as follows. Sheep erythrocytes were washed 3 times and incubated with the same volume of mouse anti-sheep erythrocyte serum (amboceptor), diluted to give a final concentration of 2%. After 40 min at 37°C the cells were washed, brought to a 4% suspension and then mixed with an equal volume of appropriately diluted goat-anti-mouse gamma globulin serum. After incubation at 37°C for 60 min, the cells were washed 3 times and made up to a 4% suspension. The indicator cells were prepared freshly every week.

The target cells in the microtiter wells were washed twice and incubated with 0.01 ml of the appropriate dilution of antiserum. All antisera were tested at dilutions of 3, 10, 30, 100, 300, 1 000 and 3 000. After 60 min incubation at room temperature, the plates were washed 3 times and 0.01 ml of a 0.5% indicator cell suspension was added. In all experiments two different concentrations of amboceptor and anti-mouse gamma globulin serum were used: 1 200 and 50, and 1 800 and 30, respectively. The plates were left at room temperature for 60 min to allow the indicator cells to settle. They were then inverted and left in this position for 30–60 min.

The strength of the reaction was evaluated under the microscope and graded as follows. +++ : all target cells are covered by a heavy layer of sensitized erythrocytes; ++ : almost all target cells show attached erythrocytes, most of them show complete rosettes; + : 50–100% of the cells show 3 or more attached erythrocytes and some complete rosettes; ± : less than 50% of the cells have 2 or more attached erythrocytes; – : no attached erythrocytes.

Infectivity assay

2.5×10^5 cells were seeded in milk dilution bottles in Eagle's Minimal Essential Medium containing 10% fetal calf serum. The medium was collected after 24 h growth. Ascites tumor cells, grown *in vivo*, were tested for release of virus in the following way. The ascitic fluid was removed and the cells were washed through 3 cycles of centrifugation in Eagle's medium containing 10% fetal calf serum. 10^6 cells in 2 ml of the medium were incubated at 37°C for 4 h and then spun out at 1 500 g for 10 min. One ml samples of the supernatant were assayed for infectivity on JLS-V9 cells [17], seeded into bottles 5 h previously. The JLS-V9 cells were subcultured at a 1:10 dilution twice a week. The cells were tested for Moloney virus-specific membrane fluorescence each week. Positive tests were repeated at least once. Negative cultures were carried for 5 weeks and then discarded.

RESULTS

Chromosomal constitution of parent and hybrid cells

The hybrid nature of the YAC-A9 and YACIR-A9 cells was confirmed by karyo-

typic analysis. Since the YACIR cells lack chromosomal markers and the single bi-armed chromosome of the YAC cells does not differ from those of the A9 cells, the hybrid metaphases were identified by the total chromosome number, the number of bi-armed chromosomes (24–28) and the presence of other markers (2 dot chromosomes and 1 very small metacentric element) derived from the A9 parent [2].

Fig. 1a shows the chromosomal constitution of the parent cells. The modal chromosome number of A9 is 56, with a range of 53–58. This number includes 26 bi-armed chromosomes. Both YAC and YACIR have a diploid mode (40 chromosomes) with a narrow range (39–41 and 40–42, respectively). One bi-armed chromosome was seen in 84% of the YAC cells, whereas the YACIR cells contained none. The chromosomal constitution of the hybrid cells is shown in fig. 1b. The modal chromosome number of YAC-A9 was 88, ranging from 83 to 103; YACIR-A9 had a mode of 92 and a range of 78–94. The hybrids thus had slightly lower modal numbers than the expected sum of the two parental modes (96). YAC-A9 had 26 bi-armed chromosomes, YACIR-A9 27; all other A9 markers were present in both hybrids.

Six per cent of the YAC-A9 and 12% of the YACIR-A9 metaphases were polyploid, with chromosome numbers ranging between 162–177 and 183–193 respectively; these polyploid cells contained approximately twice the number of bi-armed chromosomes present in one A9 cell.

Growth characteristics of hybrid cells

Both YAC-A9 and YACIR-A9 hybrids had a “fibroblastic” morphology and growth character, although the pattern of growth of the hybrid cells was less regular than that of A9 cells. In these hybrids “fibroblastic”

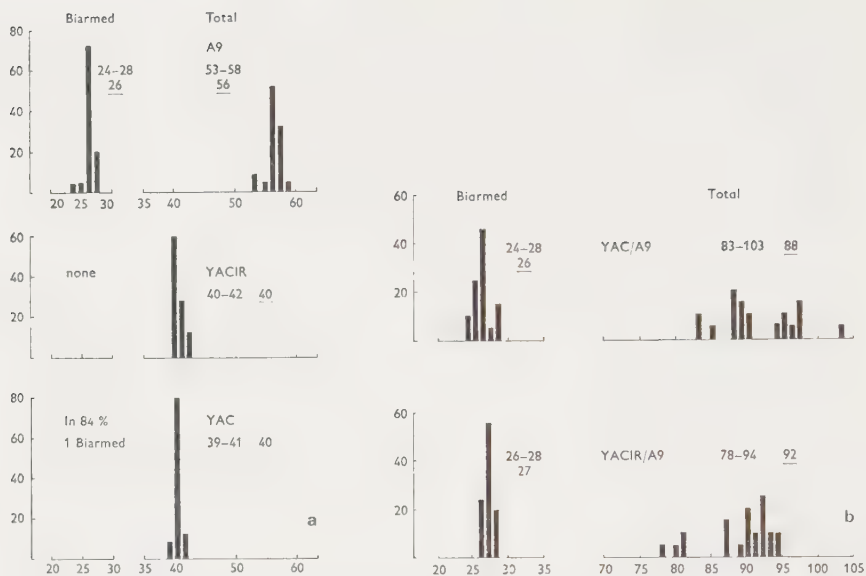


Fig. 1. Abscissa: number of chromosomes; ordinate: % cells showing indicated chromosome number. Chromosomal constitution of (a) A9, YAC and YACIR parent cells and (b) YAC-A9 and YACIR-A9 hybrid cells. Numerals denote the range and mode (underlined) of chromosome number.

morphology was thus "dominant" over lymphoid morphology. This apparent dominance was not simply due to loss of the YAC or YACIR chromosomes that determine the lymphoid morphology; for lymphoid morphology sometimes reappeared in segregant tumours arising from these YAC-A9 and YACIR-A9 hybrids. These tumours showed substantial losses of the chromosomes derived from the A9 parent cell. Work is in progress with the aim of presenting a detailed analysis of these morphological segregants. It is possible that some YAC-A9 or YACIR-A9 hybrids might have a lymphoid morphology, but such cells would not be expected to adhere to the surface of the culture vessel and would therefore be eliminated together with the YAC and YACIR parent cells.

Moloney virus-specific surface antigens and release of virus in YAC and YACIR lymphoma cells

The sensitivity of the two lymphoma lines to the cytotoxic effect of anti-Moloney serum was measured 6 weeks prior to cell fusion. The lines behaved as previously described [4]: YACIR cells were almost completely resistant to the antiserum, whereas YAC was highly sensitive (fig. 2a). Both lines were equally sensitive to H-2^a antibodies (fig. 2b). As shown previously [4], both lines reacted with anti-Moloney serum in indirect membrane immunofluorescence tests, but the YACIR line gave a lower endpoint (by 5 twofold dilution steps), thus showing that the difference between the two sublines was quantitative rather than qualitative (fig. 3c).

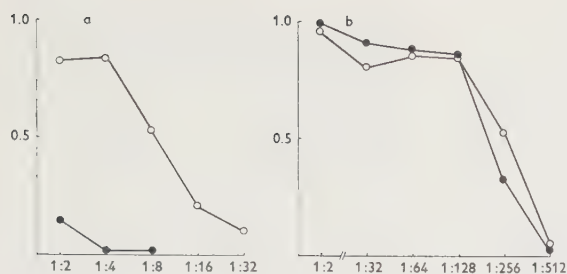


Fig. 2. Abscissa: serum dilution; ordinate: cytotoxic index.

Comparison of the cytotoxic sensitivity of the YAC line and its immunoresistant subline (YACIR) to (a) anti-Moloney and (b) anti-H-2^d (ASW anti A) sera.

The virus release test [6] showed that the YAC cells released highly infectious Moloney virus on 4 h incubation *in vitro*, while the YACIR culture did not release detectable amounts (fig. 4).

Expression of parental surface antigens in the hybrid cells

Ninety to hundred percent of both hybrid lines gave positive membrane immunofluorescence reactions with anti-H-2^k serum (fig. 3a). The antigens of the H-2^k complex are present on both parent cells. The close similarity of the titration curves was also confirmed by mixed hemadsorption (cf. table 1), which shows that YAC-A9 and YACIR-A9 cells have approximately equal amounts of H-2^k antigens on their surfaces. The H-2^d antigen complex is present only in the YAC and the YACIR cells, and it serves as a marker for the identification of the hybrid lines. As illustrated in fig. 3b, 90–100% of both hybrid cells reacted with anti-H-2^d serum, whereas the A9 cells failed to react. A corresponding pattern was obtained in the mixed hemadsorption test (table 1).

The lymphoma cells did not react with anti-L serum as judged by membrane immunofluorescence (fig. 3d). Mixed hem-

adsorption could not satisfactorily be done, however, since lymphoma cells do not adhere to the surface of the culture vessel. The L antigen on the surface of the hybrid cells must therefore have been derived from the A9 parent. As shown in fig. 3d and in table 1, both hybrid lines carried L antigen in concentrations comparable to those found in the A9 cells. Only about 50% of each cell type showed this antigen in fluorescence tests.

The difference between the YAC and YACIR lymphomas in the degree of expression of the Moloney-specific surface antigen was abolished in the YAC-A9 and YACIR-A9 hybrids (fig. 3c and table 1). Nearly 100% of the cells of both hybrid lines showed positive membrane immunofluorescence and both lines gave identical titration curves; only 50% of the A9 cells gave positive membrane fluorescence tests.

In short, the YAC-A9 and YACIR-A9 hybrids possess all the surface antigens seen in the parental cells (table 2), and the quantitative expression of these antigens is closely similar in both hybrid lines.

Release of infectious virus by parent and hybrid cells

The induction of membrane immunofluorescence in negative indicator cells (JLS-V9) by

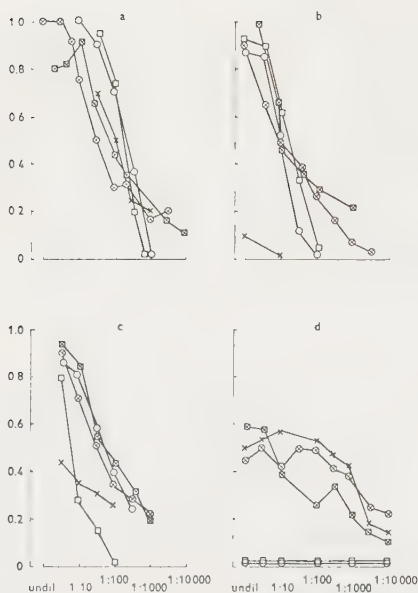


Fig. 3. Abscissa: serum dilution; ordinate: fluorescence index. $\circ$, YAC; $\square$, YACIR; $\times$, A9; $\odot$, YAC-A9; and $\boxtimes$, YACIR-A9.

Reactivity of (a) C57BlxDBA/2 anti C3H (anti-H-2^b); (b) C57BlxC3H anti DBA/2 (anti-H-2^d); (c) ABYxDBA/2 anti YDYA (anti-Moloney), and (d) C3HxDBA/2 anti A9 (anti-L) serum with parent and hybrid cells in indirect membrane immunofluorescence tests. Each point represents the mean of 3-4 individual tests with (a), (c) and (d) sera and 2 tests with (b) serum.

culture supernatants was used as the basis of the virus assay [17]. Serial dilutions of culture supernatant from the parent and hybrid cells were tested. The JLS-V9 cultures were examined each week for the appearance of the Moloney-virus-induced surface antigen. The results are shown in fig. 4. As shown previously, YAC cells released infectious virus while YACIR cells did not. A9 cells also released virus, demonstrable even at a 10^{-3} dilution of the culture fluid. The YAC-A9 hybrid released infectious virus in amounts comparable to those released by the

parent cells. No infectious virus could be demonstrated in the medium of the YACIR-A9 cultures. At the dilutions of medium tested, this means that there was at least a thousandfold difference in the infectivity of the medium from the two hybrid lines.

DISCUSSION

In a previous study on the antigenic characteristics of hybrid cells [12], it was found that

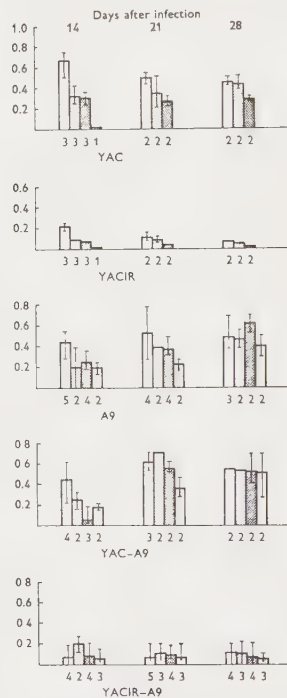


Fig. 4. Ordinate: fluorescence index. Medium dilutions: $\square$ undiluted, $\square$ 10^{-1} , $\square$ 10^{-2} , and $\square$ 10^{-3} .

JLS-V9 cells were infected with media derived from parent (YAC, YACIR and A9) and hybrid (YAC-A9 and YACIR-A9) cell cultures. Appearance of Moloney-specific surface antigen was followed by membrane immunofluorescence. Numbers of repeated experiments are indicated under the columns and the range of the results is shown by the bars at the top of each column.

Table 1. Reactivity of parent and hybrid lines in the mixed hemadsorption test

Serum dilutions reciprocal	Antigen detected	Cell lines		
		Parent A9	Hybrids	
			YAC-A9	YACIR-A9
C57BlxDBA/2 anti C3H				
10	H-2 ^k	+	+	+
30		+	+	+
100		+	+	+
300		+	+	+
1 000		+	+	+
3 000		-	+	+
C57BlxC3H anti DBA/2				
10	H-2 ^d	-	+	+
30		-	+	+
100		-	+	+
300		-	-	+
1 000		-	-	-
3 000		-	-	-
ABYxDBA/2 anti YDYA				
3	Moloney	+	+	+
10		+	+	+
30		+	+	+
100		+	+	+
300		+	+	+
1 000		-	-	+
C3HxDBA/2 anti A9 L				
10	L	+	+	+
30		+	+	+
100		+	+	+
300		+	+	+
1 000		+	+	+
3 000		+	+	+
10 000		-	+	+

Evaluation of strength of reaction: +++ - all target cells surrounded by a heavy layer of sensitized erythrocytes; ++ almost all target cells show attached erythrocytes, most in complete rosettes; + 50-100% of the cells show 3 or more attached erythrocytes, some complete rosettes; less than 50% of the cells have 2 or more erythrocytes sticking to their surface; - no erythrocytes. The data in this table are based on 3-4 independent experiments. The lymphoma cells do not adhere to the culture vessel and therefore mixed hemadsorption could not readily be done with them.

Ehrlich ascites tumour cells, selected through many decades of homotransplantation for reduced expression of membrane isoantigens, suppressed the isoantigens contributed to the hybrid cell by the other parent cell. The question arose whether this form of antigenic suppression was limited to the Ehrlich tumour, or whether other tumour lines, selected for diminished expression of specific virus-induced membrane antigens, might behave similarly. The YAC and YACIR Moloney lymphoma lines appeared to be particularly suitable for such a study. YACIR was selected from YAC by serial passage in syngeneic hosts immunized against Moloney virus. YACIR is completely resistant to the cytotoxic effect of anti-Moloney serum and has a diminished expression of the Moloney-specific surface antigen [4]. The release of infectious Moloney virus by the YACIR cells was also greatly reduced relative to the YAC cells [6]. The infectivity assay, based on the induction of Moloney-specific membrane immunofluorescence in the JLS-V9 indicator cells showed that the difference was 10⁴-fold. If the number of budding and extracellular particles is considered, as detected by electron microscopy, the difference was approximately tenfold. This indicates that the majority of YACIR particles are non-infectious.

In both the YAC-A9 and YACIR-A9 hybrids all the surface antigens seen in the two parent cells were expressed. The A9 cell contributed H-2^k isoantigens, the L-antigen and a Moloney-type antigen. The YAC cell contributed the H-2^d isoantigen complex, which is composed of H-2^k and H-2^d components, and a fully expressed Moloney-specific surface antigen; the YACIR cell contributed the same isoantigens as YAC and the Moloney-type antigen at a much lower concentration. The hybrid cells showed H-2^k and H-2^d isoantigens, L antigen and Moloney-type antigen, all fully expressed. There was no distinction

Table 2. *Expression of parental surface antigens in the hybrid cells*

Cell type	Presence of antigen		
	H-2 ^k	H-2 ^d	Moloney L
YAC ^a	+	+	+
YACIR ^a	+	+	+
A9 ^b	+	+	+
YAC-A9 ^b	+	+	+
YACIR-A9 ^b	+	+	+

^a Based on the results of cytotoxic and membrane immunofluorescence tests (cf fig. 2a and fig. 3a-d).

^b Based on the results of membrane immunofluorescence and mixed hemadsorption tests (cf fig. 3a-d and table 1).

between YAC-A9 and YACIR-A9 lines in the degree to which they permitted the expression of the Moloney-type, or any other surface antigen. The YACIR cell thus behaved very differently from the Ehrlich cell, when fused with the A9 cell. The Ehrlich cell, which has mechanisms for the suppression of its own surface antigens, suppressed the surface antigens (both H-2^k and L) introduced into the hybrid by the A9 cell; whereas the YACIR cell, which has the ability to suppress Moloney virus-induced surface antigen, loses this ability when fused with the A9 cell. In the case of the Ehrlich-A9 hybrids, antigenic suppression is thus "dominant"; in the YACIR-A9 hybrids, it is "recessive". The difference between the two cases is probably due to the fact that, in one case (Ehrlich), selection has been against the expression of isoantigens, whereas, in the other, (YACIR) only a specific virus-induced antigen has been selected against. Thus the underlying mechanisms for decreased antigen expression may be completely different. The further study of hybrids between cells subjected to different kinds of immune selection, and especially between sublines of the same tumour, selected for reduced expression of different surface antigens might help to illuminate the mech-

anisms of antigenic suppression, and hence the phenomenon of immunoresistance in tumours.

Like the parental lymphoma cell, the YACIR-A9 hybrid did not release detectable amounts of infectious Moloney virus. This indicates that the production of the specific surface antigen and the production of infectious virus are not coupled, a conclusion in agreement with previous studies [10]. Whether non-infectious particles were formed in the YACIR-A9 hybrid was not investigated. Such particles may be formed and their production might be reflected by full expression of the membrane antigen. Alternatively, the production of the virus-induced membrane antigen may not be directly coupled to the synthesis of C-type particles. It is of interest to note that C-type particles, infectious for the assay system, are released by the A9 cell, but not by the YACIR-A9 hybrid. This suggests that the mechanisms suppressing the release of infectious virus in the YACIR cell are dominant in the hybrid cell and that they act at some point in the viral cycle subsequent to the formation of the virus-specific cell surface antigen.

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